The role of retinoic acid in long-term memory formation and synaptic plasticity in the mollusc *Lymnaea stagnalis*

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Abstract

The active metabolite of vitamin A, retinoic acid (RA), is involved in memory formation and hippocampal plasticity in vertebrates. A similar role for retinoid signaling in learning and memory formation has not previously been examined in an invertebrate species. However, the conservation of retinoid signaling between vertebrates and invertebrates is supported by the presence of retinoid signaling machinery in invertebrates. For example, in the mollusc *Lymnaea stagnalis* the metabolic enzymes and retinoid receptors have been cloned from the CNS. In this study I demonstrated that impairing retinoid signaling in *Lymnaea* by either inhibiting RALDH activity or using retinoid receptor antagonists, prevented the formation of long-term memory (LTM). However, learning and intermediate-term memory were not affected. An additional finding was that exposure to constant darkness (due to the light-sensitive nature of RA) itself enhanced memory formation. This memory-promoting effect of darkness was sufficient to overcome the inhibitory effects of RALDH inhibition, but not that of a retinoid receptor antagonist, suggesting that environmental light conditions may influence retinoid signaling. Since RA also influences synaptic plasticity underlying hippocampal-dependent memory formation, I also examined whether RA would act in a trophic manner to influence synapse formation and/or synaptic transmission between invertebrate neurons. However, I found no evidence to support an effect of RA on post-tetanic potentiation of a chemical synapse. Retinoic acid did, however, reduce transmission at electrical synapses in a cell-specific manner. Overall, these studies provide the first evidence for a role of RA in the formation of implicit long-term memories in an invertebrate species and suggest that the role of retinoid signaling in memory formation has an ancient origin.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ADH	Alcohol dehydrogenase
ANOVA	Analysis of variance
atRA	All-*trans* retinoic acid
cDNA	Complimentary deoxynucleic acid
CNS	Central nervous system
CPG	Central pattern generator
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
Cyp26	Cytochrome P450 protein 26
DEAB	4-diethylaminobenzaldehyde
DM	Defined medium
DMSO	Dimethyl sulfoxide
EIF4α	Eukaryotic initiation factor 4α
EPSP	Excitatory postsynaptic potential
EtOH	Ethanol
IP3	Input 3 Interneuron
ITM	Intermediate-term memory
KCl	Potassium chloride
LPeD1	Left Pedal Dorsal 1
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
mRNA	Messenger ribonucleic acid
MT	Memory test
PCR	Polymerase chain reaction

PW	Pond water
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RPeD1	Right Pedal Dorsal 1
RPeD2	Right Pedal Dorsal 2
RXR	Retinoid X receptor
SEM	Standard error of the mean
STM	Short-term memory
VAD	Vitamin A deficient
VD4	Visceral Dorsal 4
VH/VK	Visceral H/Visceral K cells (Pneumostome closer motorneurons)
VI/VJ	Visceral I/Visceral J cells (Pneumostome opener motorneurons)

Chapter 1:

Introduction and Literature Review

1.01 General introduction

Retinoic acid (RA) is the active metabolite of vitamin A and is essential for vertebrate development by influencing patterning, neuronal differentiation (Maden, 2002, 2007), and neurite outgrowth (Corcoran et al., 2000; Dmetrichuk et al., 2005; Clagett-Dame et al., 2006). However, RA is not exclusively active during development and is also active in the adult nervous system (Maden, 2007). Synthesis of RA occurs in the adult brain (Dev et al., 1993; Werner and DeLuca, 2002), and RA has been detected in brain regions such as the cortex, cerebellum, and hippocampus (Kane et al., 2005). Retinoid signaling machinery has also been detected within the vertebrate hippocampus (Krezel et al., 1999; Zetterström et al., 1999; Fragoso et al., 2012; Goodman et al., 2012), and RA is involved in hippocampal-dependent memory formation (Chiang et al., 1998; Misner et al., 2001; Wietryzch et al., 2005; Nomoto et al., 2012). While retinoid signaling machinery has been identified in invertebrates (Cañestro et al., 2006; Albalat and Cañestro, 2009), and RA influences embryonic development (Carter et al., 2010, 2015) and neuronal outgrowth and survival (Dmetrichuk et al., 2006, 2008), similar to its role in vertebrates, no studies have previously investigated whether retinoid signaling is involved in learning and memory in an invertebrate species.

This research explores the conservation of retinoid signaling between vertebrates and invertebrates by examining the role of RA in memory formation, using the mollusc *Lymnaea stagnalis.* First, I used behavioural observations to examine whether disruptions in retinoid signaling influenced learning and/or the formation of memory in the mollusc *L. stagnalis*. Having determined that RA was required for long-term memory (LTM) formation, I then utilized identified neurons in cell culture to determine whether RA exerted trophic influences on synaptogenesis or synaptic plasticity. By investigating the role of RA in memory formation in an

invertebrate species, these studies shed light on the origin of retinoid signaling and the role of RA in memory formation.

1.02 Retinoic acid synthesis and signaling

Vitamin A (retinol) is a fat soluble vitamin which must be obtained via dietary means from either plants (in the form of carotenoids) or animal products (in the form of retinyl esters) (Maden, 2007; Theodosiou et al., 2010). While the canonical RA synthesis pathway has been greatly studied, alternative ancestral synthesis pathways have recently been described (Simões-Costa et al., 2008).

In the canonical pathway, retinol (vitamin A) is ingested and stored in the form of retinyl esters. Retinol binds to retinol-binding proteins (RBPs) in the bloodstream for transportation to various target regions. The retinol enters target cells via the vertebrate RBP receptor STRA6 (stimulated by retinoic acid gene 6) and can then associate with cellular retinol binding proteins (CRBPs) within the cytoplasm for transportation throughout the cell (Maden, 2007; Theodosiou et al., 2010). Retinol can next be oxidized to the active metabolite RA via a two step enzymatic reaction. In the first step, retinol is converted into retinal via an alcohol dehydrogenase (ADH) enzyme. This newly formed retinal is then converted to RA via an irreversible oxidation reaction involving a retinaldehyde dehydrogenase (RALDH) enzyme (Maden, 2007; Theodosiou et al., 2010). Many isoforms of RA exist, but the two major isoforms are all-*trans* RA (atRA) and 9-*cis* RA (Maden, 2002). The canonical RA synthesis pathway is illustrated in Figure 1 (in black).

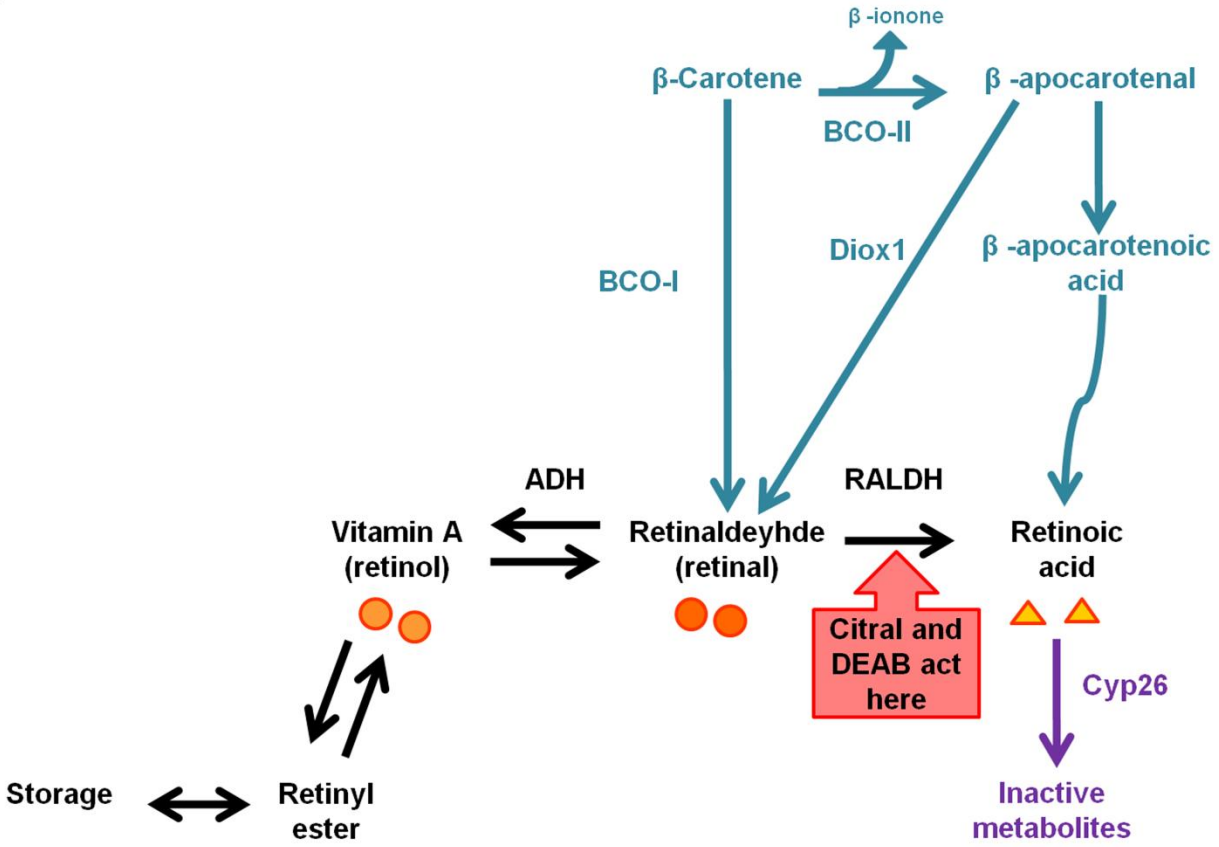

Figure 1. Canonical and alternative retinoid signaling pathways. In the canonical RA synthesis pathway (black) vitamin A (retinol) is oxidized via a two step metabolic reaction catalyzed by ADHs and RALDHs. In these studies citral or DEAB were used to inhibit RALDH enzyme activity. In an alternative, ancestral pathway (blue), β-carotene is symmetrically cleaved to produce retinaldehyde (retinal), which can then be oxidized to RA. β-carotene may also be asymmetrically cleaved, and the β-apocarotenal produced can either be converted into retinaldehyde (retinal) or β-apocarotenoic acid, which in turn is oxidized to RA. The Cyp26 enzymes are responsible for the breakdown of RA into inactive metabolites. (Modified from Simões-Costa et al., 2008).

Alternative, ancestral pathways for RA synthesis have been proposed which do not involve the oxidation of retinol via ADH enzymes and instead process β-carotene. The β,β-carotene-15', 15'- monooxygenase enzyme (BCO-I) symmetrically cleaves β-carotene to produce two all-*trans*-retinaldehyde molecules which can then be oxidized to RA by RALDH enzymes (Simões-Costa et al., 2008; Theodosiou et al., 2010). A second enzyme, β,β-carotene-9', 10'- dioxygenase (BCO-II), also cleaves β-carotene, but it does so asymmetrically to produce β-apocarotenal and β-ionone (Simões-Costa et al., 2008; Theodosiou et al., 2010). β-

apocarotenal can then either be cleaved to produce a retinaldehyde molecule which is subsequently converted to RA (Simões-Costa et al., 2008), or it can be converted to β-apocarotenoic acid which is then oxidized to RA (Simões-Costa et al., 2008; Theodosiou et al., 2010). Thus, the canonical and most of the ancestral RA synthesis pathways require the final oxidation of retinaldehyde to RA via RALDH enzymes, but an alternative pathway which does not require retinaldehyde has also been proposed. The ancestral synthesis pathways are illustrated in blue in Figure 1.

Once synthesized, RA associates with cellular retinoic acid binding proteins (CRABPs) within the cytoplasm and is transported either within the cell (to participate in autocrine signaling) or outside of the cell (to participate in paracrine signaling) (Maden, 2007). The CRBPs and CRABPs regulate the retinoid signal by buffering the concentration of free retinoids within the cytoplasm, as too much or too little RA can lead to developmental defects as well as abnormalities within the adult nervous system (McCaffery et al., 2003). Endogenous RA is catabolised via the action of the Cyp26 family of enzymes (cytochrome P450 enzymes) to produce oxidized and reportedly inactive products (Theodosiou et al., 2010).

Retinoic acid classically acts to regulate gene transcription via binding to retinoid receptors, which are members of the nuclear hormone receptor family, and act as transcription factors to regulate the expression of target genes (Maden, 2007; Theodosiou et al., 2010). Two classes of retinoid receptors exist in vertebrates: retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Mey and McCaffery, 2004). Three different RAR subtypes (α, β, γ) and three different RXR subtypes (α, β, γ) exist in vertebrates. Retinoic acid receptors (RARs) bind both all-*trans* RA and 9-*cis* RA, while RXRs demonstrate a preference for 9-*cis* RA, at least in vertebrates (Lane and Bailey, 2005; Theodosiou et al., 2010). Following the binding of RA, the RARs heterodimerize with RXRs or the RXRs homodimerize and bind to retinoic acid response

elements (RAREs), located in the promoter region of target genes in order to influence gene transcription (Lane and Bailey, 2005) (Figure 2).

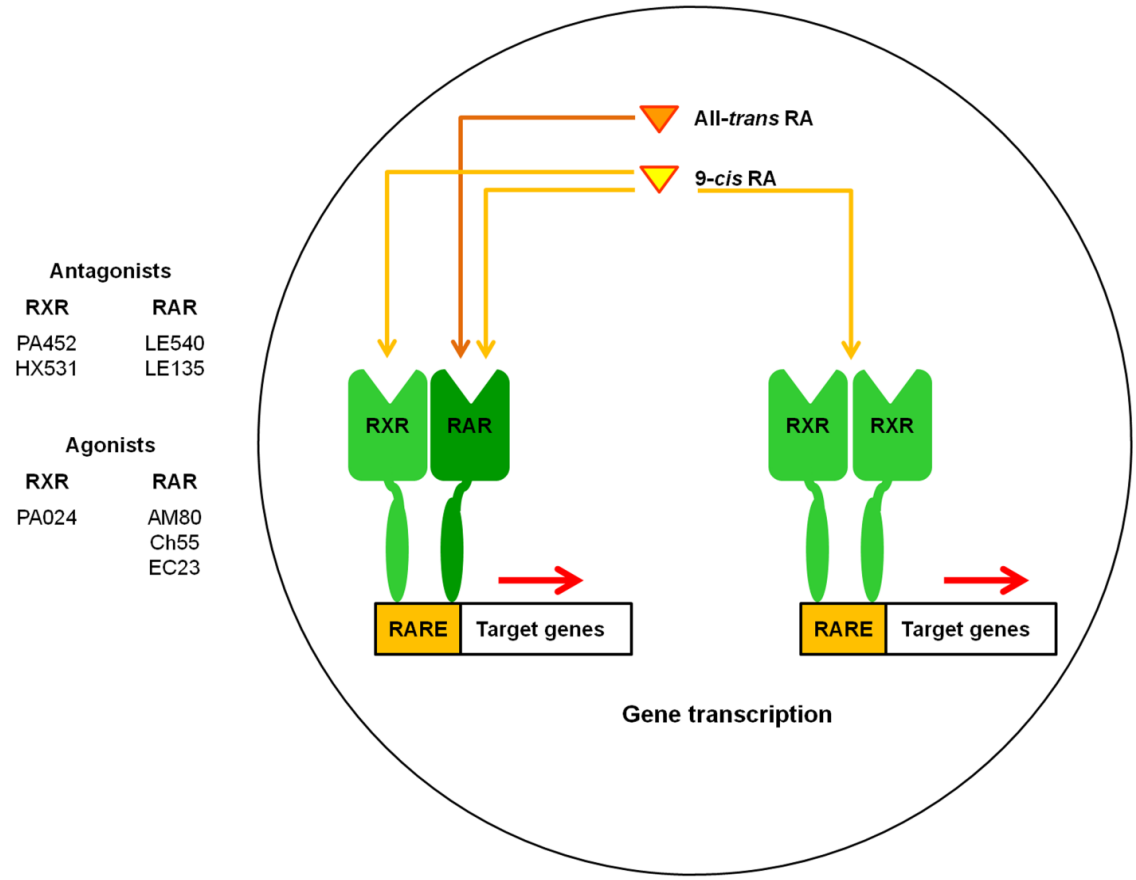

Figure 2. Regulation of gene expression by retinoid receptors. RA classically acts to regulate gene transcription by binding with nuclear RARs and RXRs. Following the binding of RA, these receptors heterodimerize or homodimerize and bind to a RARE sequence in a target gene to induce transcription. Vertebrate RXRs demonstrate a preference for 9-*cis* RA while RARs bind both 9-*cis* and all-*trans* RA. Various agonists and antagonists of the vertebrate RXR and RAR were used in these studies and are shown above. (Modified from Simões-Costa et al., 2008).

1.03 Retinoid signaling in the vertebrate nervous system

Retinoid signaling is involved in both the development and maintenance of the vertebrate nervous system. In the developing nervous system, RA influences neuronal patterning and differentiation. Specifically, retinoid signaling is involved in both anterior-posterior and dorsoventral patterning of the nervous system, with an RA gradient guiding patterning within the

developing tissue (reviewed by Maden, 2002, 2007). Retinoid signaling has also been implicated in limb development, as pharmacologically impairing RA synthesis impaired chick limb development (Tanaka et al., 1996) and prevented the development of pectoral fins in zebrafish (Vandersea et al., 1998). Interestingly, both of these defects were reversed following exogenous application of RA. Impairing RA synthesis in zebrafish also resulted in the formation of smaller eyes with significant developmental abnormalities (Le et al., 2012), while exogenous application of RA to zebrafish embryos led to the duplication of the retina (Hyatt et al., 1992) and alterations in the rate of photoreceptor development (Hyatt et al., 1996).

Retinoic acid has also been implicated in the regeneration of the adult nervous system. Following spinal cord injury in rats, RALDH activity was increased at the site of lesioning (Mey et al., 2005). Additionally, caudal spinal cord and tail regeneration were reduced in the newt *Notopthalmus viridescens* when retinoid signaling was impaired using an RARβ-selective antagonist (Carter et al., 2011), and cultured *N. viridescens* spinal cord explants showed enhanced neurite outgrowth in the presence of RA, which was also inhibited by the RARβ-selective antagonist (Dmetrichuk et al., 2005).

The distribution of retinoid machinery in the adult brain also implicates retinoid signaling in the cognitive processes of learning and memory. Retinoic acid is synthesized in the adult nervous system (Dev et al., 1993; Werner and DeLuca, 2002) and has been detected throughout the brain, including the hippocampus (Werner and DeLuca, 2002; Kane et al., 2005; Goodman et al., 2012), which is an area known to be important for learning and memory formation. Retinoid signaling machinery, including binding proteins which regulate cellular retinoid levels (CRBPs and CRABPs; Zetterström et al., 1994; Goodman et al., 2012), the RA synthesizing enzyme RALDH (Fragoso et al., 2012; Goodman et al., 2012) and retinoid receptors (both RARs and RXRs) are present throughout the adult nervous system, including (but not limited to) the

hippocampus (Krezel et al., 1999; Zetterström et al., 1999; Fragoso et al., 2012). Together, these data indicate that retinoid signaling is active within the adult nervous system.

1.04 Retinoid signaling in invertebrates

Previously, retinoid signaling was thought to occur predominantly in chordate species and it was widely accepted that chordates possessed both RARs and RXRs, while non-chordate invertebrates were thought to only possess RXRs. However, Cañestro et al. (2006) showed for the first time that enzymes responsible for RA synthesis (ALDHs) and degradation (Cyp26), as well as RARs are present in non-chordate deuterostomes, indicating that retinoid signaling was not a chordate innovation, as previously thought. Retinoid signaling machinery (ALDH, Cyp26, and RAR) has been shown to be present in protostomes (including the mollusc *Lottia gigantea*), indicating that at least some protostome species retained the genetic components of retinoid machinery (Albalat and Cañestro, 2009). Endogenous retinoids have also been detected in protostomes (Dmetrichuk et al., 2008; Nowickyj et al., 2008; Gesto et al., 2012), and protostome retinoid receptors (RXRs) have been shown to bind RA (Bouton et al., 2005; Nowickyj et al., 2008).

An RXR (*Lym*RXR; Carter et al., 2010; Genbank Accession no. AY846875) and a putative RAR (*Lym*RAR; Carter et al., 2015; Genbank Accession no. GU932671), as well as the enzyme involved in RA synthesis, RALDH (*Lym*RALDH; Genbank Accession no. FJ539101) and the enzyme involved in RA degradation, Cyp26 (*Lym*Cyp26; Genbank Accession no. KF669878), have all been cloned from the *Lymnaea* CNS. Both the *Lymnaea* CNS and hemolymph contain all-*trans* RA and 9-*cis* RA, and RALDH activity has been demonstrated in *Lymnaea* CNS extracts (Dmetrichuk et al., 2008).

Numerous actions of retinoid signaling observed in chordates have also been observed in protostomes. For instance, in the fiddler crab *Uca pugilator*, limb regeneration was disrupted by exposure to RA (Hopkins and Durica, 1995). In the mollusc *Lymnaea stagnalis*, disruptions in retinoid signaling impaired embryonic development, resulting in eye defects, shell malformations, and halted development (Créton et al., 1993; Carter et al., 2010, 2015). Retinoic acid also influenced neuronal outgrowth and survival in adult *Lymnaea* neurons (Dmetrichuk et al., 2006, 2008), similar to its observed roles in vertebrates (Corcoran et al., 2000, 2002; Clagett-Dame et al., 2006). A role for RA as a chemoattractant has also been shown in *Lymnaea*, as regenerating growth cones turn toward a source of RA in culture (Dmetrichuk et al., 2006, 2008; Farrar et al., 2009). Taken together, these observations suggest that other roles of retinoid signaling observed in chordates (such as its involvement in memory formation) may also be conserved in a protostome species.

1.05 Learning and memory formation

In order to survive, a species must be able to adapt its behaviour to ever changing environmental conditions, which is the basis of learning and memory (Lukowiak and Syed, 1999). Learning refers to the acquisition of new information, while memory involves encoding, storing, and retrieving learned information (Lukowiak and Syed, 1999; Kandel et al., 2014).

Learning can be described as either nonassociative or associative. In nonassociative learning, an individual alters its behaviour following repeated exposure to a single stimulus. Nonassociative learning is typically classified as either habituation (in which there is a reduced response) or sensitization (in which a response is amplified). Associative learning involves the formation of an association between two different events or stimuli and can be generated by

either classical or operant conditioning. In classical conditioning, a neutral stimulus (the conditioned stimulus) is repeatedly paired with a biologically relevant stimulus (the unconditioned stimulus), and over time the animal forms an association between the two previously unrelated stimuli. Operant conditioning involves the formation of an association between the performance of a specific behaviour and the outcome (or consequence) of that behaviour, which then modifies future behaviour (Carew and Sahley, 1986).

Memory can be sub-defined in terms of its duration. Short-term memory (STM) refers to information that remains in our consciousness for only a few minutes, intermediate-term memory (ITM) can persist for a few hours, and long-term memory (LTM) persists for many hours, days, and even years. The mechanisms underlying formation of STM, ITM and LTM are different, with LTM formation requiring both transcription and translation, ITM requiring only translation, and STM requiring neither (Crow et al., 1999; Sangha et al., 2003a; Kandel et al., 2014).

1.06 Retinoic acid is involved in vertebrate memory formation

Retinoid signaling has been implicated in vertebrate memory formation, with disruptions in retinoid signaling resulting in impaired memory in rodents. Additionally, avian studies demonstrated that RA is required for vocal and auditory learning in the zebra finch. Studies in rodents have used two different approaches to assess the role of RA (and retinoid signaling) in learning and memory: a) maintaining animals on a vitamin A deficient (VAD) diet to prevent RA synthesis and b) the use of retinoid receptor mutants. Rodents maintained on a VAD diet demonstrated impairments in both long-term potentiation (LTP) and long-term depression (LTD; Misner et al., 2001), two forms of synaptic plasticity thought to underlie learning and memory formation. A reduction in the size of the CA1 hippocampal region (Cocco et al., 2002), reduced

retinoid receptor (RARβ and RXRβ/γ) expression (Etchamendy et al., 2003), and decreased cell proliferation (which was reversible following treatment with RA (Bonnet et al., 2008)) have also been reported in VAD rodents. Rats maintained on a VAD diet also demonstrated impaired spatial memory formation (Cocco et al., 2002; Bonnet et al., 2008) which was reversed following treatment with RA (Bonnet et al., 2008) or replacement of vitamin A (Cocco et al., 2002).

Retinoid receptor mutant rodents also demonstrated impaired memory and hippocampal plasticity. RARβ and RARβ/RXRγ, but not RXRγ mutant mice demonstrated an absence of LTP and severe impairments in spatial learning and memory formation when assessed with the Morris Water Maze (Chiang et al., 1998). However, Wietrzych et al. (2005) showed that RXRγ, but not RARβ, mutant mice exhibited deficits in novel object recognition and spatial working memory. This discrepancy could be related to the type of test used to assess memory formation. Dominant-negative RAR transgenic mice also displayed impaired LTP, social recognition and spatial memory, but short-term hippocampal plasticity was not affected (Nomoto et al., 2012).

Retinoid signaling has also been implicated in vocal and auditory learning in the zebra finch; various song nuclei express an RALDH and efficiently synthesize RA (Denisenko-Nehrbass et al., 2000), and retinoid receptors are expressed within the nuclei of the song control system (Jeong et al., 2005). Impairing RALDH activity within the high vocal centre of the zebra finch brain disrupted song maturation in juveniles, but did not affect the songs observed in adults (Denisenko-Nehrbass et al., 2000). Additionally, the administration of increased dietary RA to juvenile songbirds during the critical period for song learning led to the observation of more variable songs in adulthood (Wood et al., 2008).

Retinoid signaling has thus been shown to be involved in vertebrate memory formation, but no previous research has investigated whether RA plays a similar role in learning and memory formation in invertebrate species.

1.07 Studying learning and memory using invertebrate species

The complexity of the mammalian nervous system makes it difficult to attribute the learning of a specific task or the formation of a memory to a subset of neurons. Invertebrates offer the ability to study learning and memory within simpler nervous systems (typically containing only tens of thousands of neurons), making it possible to trace behavioural modifications to individual cells.

The sea slug *Aplysia* is a model organism often used to study both nonassociative and associative learning. The gill withdrawal behaviour has been used to study the behavioural and cellular mechanisms of nonassociative learning (both habituation and sensitization) and associative learning (operant and classical conditioning) (Kandel et al., 2014). The feeding behaviour of *Aplysia* can also be operantly conditioned by presenting the animal with seaweed wrapped in netting, making it impossible to eat. The animal eventually learns to spend less time attempting to consume the seaweed (Lyons, 2011). This approach is used to study the effects of photoperiod on memory formation, which are discussed later in Chapter 3.

The mollusc *Lymnaea stagnalis* is also a model organism for studying associative learning and memory formation. The feeding behaviour of *Lymnaea* can be classically conditioned using either an aversive or nonaversive approach, and the aerial respiratory behaviour can be operantly conditioned. The neural networks underlying these behaviours have been studied, and changes in neural activity as a result of conditioning have been examined (reviewed by Benjamin et al., 2000). In the studies described in this thesis, I operantly conditioned the aerial respiratory behaviour of *Lymnaea* to assess the involvement of retinoid signaling in learning and memory formation.

1.08 The aerial respiratory behaviour of *Lymnaea stagnalis*

Lymnaea stagnalis is a bimodal breather, capable of performing cutaneous respiration when submerged under water or aerial respiration at the air-water interface (Jones, 1961; Syed et al., 1991). Cutaneous respiration is favoured in well-aerated water, while *Lymnaea* demonstrate a preference for aerial respiration in hypoxic water (Jones, 1961; Syed et al., 1991). During a complete respiratory cycle, *Lymnaea* travels to the water's surface, opens its pneumostome (the breathing pore leading to the animal's lung; see Figure 3) and expels the gas contained within the lung sac, thus obtaining air from the surrounding environment, and finally closes the pneumostome. The duration of pneumostome opening varies with each cycle, and *Lymnaea* often repeats this behaviour before returning below the surface of the water (Jones, 1961; Syed et al., 1991). This behaviour is innate, as *Lymnaea* prevented from surfacing and opening the pneumostome during development still perform aerial respiration as adults (Hermann and Bulloch, 1998; Khan and Spencer, 2008). This irregular, rhythmic behaviour is controlled by an identified three neuron central pattern generator (CPG) within the central nervous system (CNS).

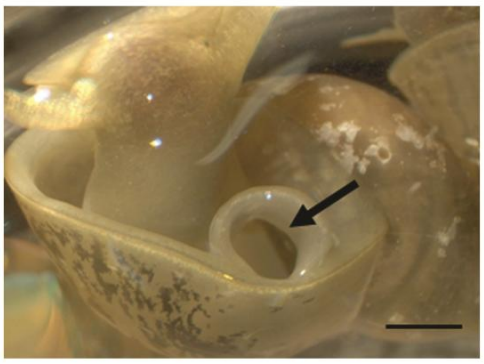

Figure 3. *Lymnaea stagnalis* opening its pneumostome and performing aerial respiration. When the snail reaches the water's surface and opens its pneumostome, gas exchange occurs with the surrounding environment. Scale bar = 3 mm. Taken from Spencer and Rothwell (2013).

1.09 The respiratory central pattern generator of *Lymnaea stagnalis*

Many of the cells distributed in the 11 ganglia of the *Lymnaea* CNS (Figure 4A) have been identified and had their network connections and functions described (Benjamin and Winlow, 1981; Winlow et al., 1981; Syed and Winlow, 1991a; Syed et al., 1991; Magoski and Bulloch, 1997). The aerial respiratory behaviour is controlled by a three neuron CPG (a neuronal network which controls rhythmic output to motorneurons and/or muscles) consisting of Right Pedal Dorsal 1 (RPeD1; a giant dopaminergic cell), Input 3 Interneuron (IP3), and Visceral Dorsal 4 Interneuron (VD4). Syed et al. (1990) demonstrated that RPeD1, IP3, and VD4 are necessary and sufficient to produce the rhythmic output required for aerial respiratory activity, by reconstructing this neuronal network in culture and showing that the observed bursting activity matched the activity observed in the CNS (Syed et al., 1990; Syed and Winlow, 1991).

Right Pedal Dorsal 1 (RPeD1) initiates the activity within the respiratory CPG, expiration (pneumostome opening) is initiated by IP3, and inspiration (pneumostome closing) is initiated by VD4 (Syed and Winlow, 1991a). The reciprocal inhibitory synaptic connections between IP3 and VD4 allow for alternate bursting within the CPG. Removal or destruction of RPeD1 (Scheibenstock et al., 2002) or VD4 (Syed et al., 1992) prevents the performance of aerial respiration, indicating the importance of these neurons to the performance of this behaviour. Chemosensory input from the hypoxic environment is conveyed to the CPG from the osphradium via RPeD1 (Inoue et al., 2001; Bell et al., 2007), and RPeD1 also receives excitatory mechanosensory input when the pneumostome breaks the water's surface (Haque et al., 2006). The motorneurons contacting the pneumostome muscle have also been identified, and pneumostome opening is controlled by the VI/VJ cells while pneumostome closing is controlled by the VH/VK cells (Syed et al., 1991). Figure 4B shows the network connections responsible for aerial respiration in *Lymnaea*.

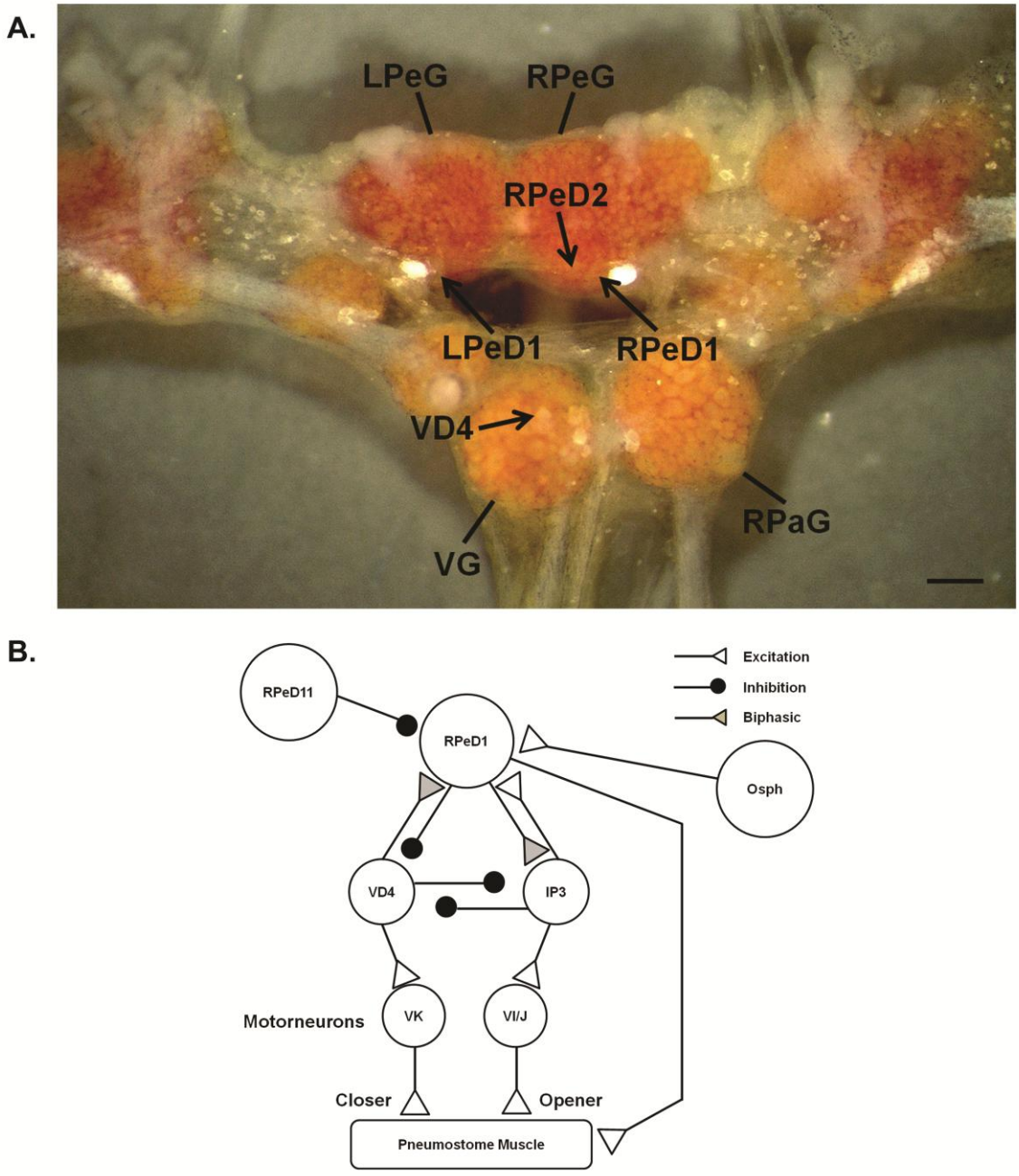

Figure 4. The *Lymnaea* central nervous system and aerial respiratory central pattern generator. (A) The central ganglia (CNS; dorsal surface shown) consists of 11 ganglia (buccal ganglia not shown). LPeG and RPeG: left and right pedal ganglia; VG: visceral ganglion; RPaG: right parietal ganglion. The arrows refer to the neurons used in these studies (LPeD1: Left Pedal Dorsal 1; RPeD1: Right Pedal Dorsal 1; RPeD2: Right Pedal Dorsal 2; VD4: Visceral Dorsal 4). Note that Input 3 Interneuron (IP3) is located on the ventral surface of the right parietal ganglion. Scale bar = 100 µm. **(B)** A schematic showing the connections of the aerial respiratory CPG. Osph represents osphradial neurons which excite RPeD1 during hypoxia. The biphasic connection from RPeD1 to IP3 is inhibition followed by excitation. The connection from VD4 to RPeD1 is depicted as biphasic (excitation followed by inhibition), but can also be purely inhibitory in nature. VK and VI/J are the motorneurons responsible for pneumostome closing and opening respectively. (Modified from Spencer and Rothwell, 2013).

1.10 *Lymnaea stagnalis* as a model for studying learning and memory

It was first shown that the aerial respiratory behaviour of *Lymnaea* can be operantly conditioned by Lukowiak et al. (1996) using the presentation of a punishing stimulus to the open pneumostome. Conditioning is conducted in hypoxic water to increase the number of pneumostome openings performed by *Lymnaea* (Lukowiak et al., 1996) and the stimulus is presented each time the pneumostome is opened over a number of training sessions. Following training, the performance of aerial respiration is reduced, indicating the formation of a learned association between pneumostome opening and the tactile stimulus (Lukowiak et al., 1996, 1998). The stimulus must be presented following each pneumostome opening, as the learned association is not formed in snails trained with a partial reinforcement schedule (Sangha et al., 2002). Yoked control animals that receive the same number of stimuli as operantly conditioned partners, only near the closed pneumostome, do not demonstrate a change in aerial respiratory activity (Lukowiak et al., 1996, 1998, 2000). Hypoxic control animals prevented from surfacing during training sessions also do not demonstrate reduced aerial respiratory activity (Lukowiak et al., 1996; Lowe and Spencer, 2006), indicating that the hypoxic training environment cannot account for the observed reduction in pneumostome opening following operant conditioning.

Lymnaea can be trained to form both intermediate-term memory (persisting for a few hours; ITM) and long-term memory (persisting for days or weeks; LTM) depending on both the number and duration of the training sessions (Lukowiak et al., 1998, 2000). The conditioned response persists longer when training sessions are spaced over time, as opposed to massed together, indicating that sufficient time is required for consolidation between training sessions in order for LTM formation to be successful (Lukowiak et al., 1998).

Electrophysiological changes within the respiratory CPG have also been associated with the conditioned response. The giant dopaminergic neuron RPeD1 initiates the aerial respiratory

response, and a higher percentage of RPeD1 neurons were quiescent in operantly conditioned animals relative to yoked controls (Spencer et al., 1999). Additionally, a decrease in synaptic transmission between RPeD1 and IP3 was observed in operantly conditioned snails. Spontaneous IP3 activity observed in RPeD1 also occurred significantly less often in operantly conditioned snails compared to yoked controls (Spencer et al., 1999). The ablation of the RPeD1 soma in live, intact snails prevented LTM formation following operant conditioning, but did not impair learning or ITM formation (Scheibenstock et al., 2002). Since LTM formation requires gene transcription, but learning and ITM formation do not (Sangha et al., 2003a), this suggests that gene transcription within RPeD1 is required for successful LTM formation in *Lymnaea* (Scheibenstock et al., 2002). RPeD1 soma ablation also prevented both extinction (Sangha et al., 2003b) and reconsolidation (Sangha et al., 2003c) of the conditioned response, further indicating that this neuron is essential for memory processes.

1.11 Studying synaptic plasticity using cell culture techniques

Memory formation is widely accepted to require neural plasticity in the form of changes in the strength of existing synapses as well as the formation of new synapses. Hippocampal long-term potentiation (LTP) and long-term depression (LTD) are two forms of synaptic plasticity underlying memory formation and are often investigated in cultured hippocampal slices or cultured neurons (Chiang et al., 1998; Misner et al., 2001). However, the complexity of the mammalian brain makes it difficult to study individual synapses and neurons. For this reason animals with less complex nervous systems, such as the molluscs *Lymnaea stagnalis* and *Aplysia*, are often used to study neural networks as well as the cellular processes involved in synaptic communication.

One advantage of using the CNS of *Lymnaea stagnalis* is that cells underlying specific behaviours (e.g. feeding and aerial respiration) have been identified, and their connectivity within the CNS has been mapped (Benjamin et al., 2000). These neurons can be plated in cell culture (Figure 5A) and used to examine the effects of various factors on synapse formation and modulation (Magoski and Bulloch, 1998; Lovell et al., 2002). However, it is difficult to assess the influence of some factors on synapse formation, as they may also influence the neurite outgrowth preceding synaptogenesis. For instance, trophic factors influence synapse formation, but they also influence neurite outgrowth. Molluscs such as *Helisoma* and *Lymnaea*, offer the advantage of being able to examine synapses in the absence of neurite outgrowth by culturing neurons in a soma-soma configuration for which neurite outgrowth is not required (Haydon, 1988; Feng et al., 1997; Hamakawa et al., 1999; Woodin et al., 1999; Figure 5B). Culturing cells in this configuration makes them easily accessible for electrophysiological recordings. Using this approach, the role of trophic factors in synapse formation can be examined in the absence of their role in neurite outgrowth. Although this soma-soma approach was first used in leech neurons (Fuchs et al., 1981, 1982), Haydon (1988) demonstrated that chemical synapses will form between two *Helisoma* neurons whose somata are juxtaposed in culture. In *Lymnaea* both excitatory and inhibitory synapses (which recapitulate those observed *in vivo*) have been reformed in the soma-soma configuration (Feng et al., 1997; Hamakawa et al., 1999; Woodin et al., 1999). Inhibitory synapse formation does not require extrinsic trophic factors (Feng et al., 1997), but excitatory synapses are reported to only form in the presence of extrinsic trophic factors (Hamakawa et al., 1999; Woodin et al., 1999). Thus, *Lymnaea* is an ideal model for studying learning and memory, as it is possible to use behavioural observations to assess memory formation and then examine the CNS for possible network changes. Retinoic acid has been shown to modulate transmission at both chemical (Aoto et al., 2008; Sarti et al., 2013) and

electrical (Weiler et al., 1999; Zhang and McMahon, 2000) synapses in vertebrates. However, a role for RA in synaptic plasticity between invertebrate neurons has not yet been described.

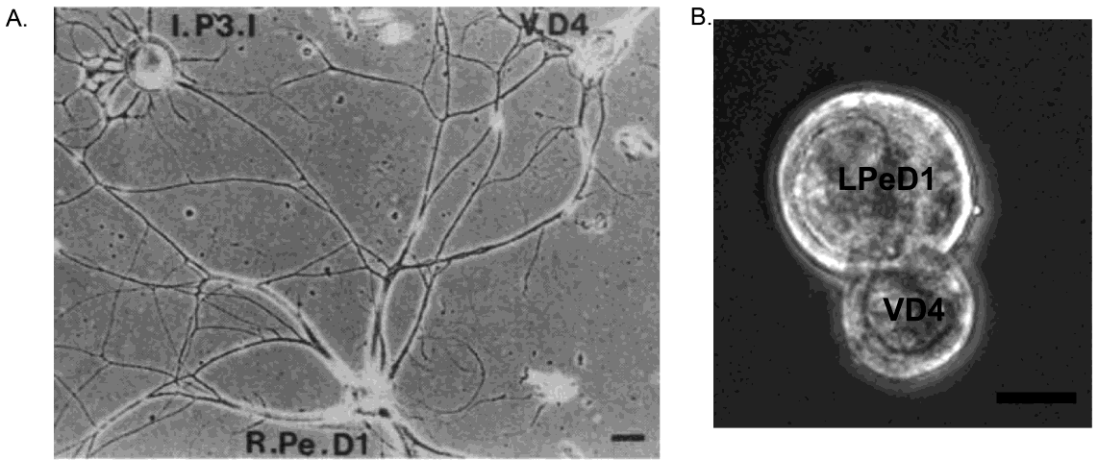

Figure 5. Synapse formation between cultured *Lymnaea* neurons. (A) The three neuron CPG underlying aerial respiration in *Lymnaea* reconstructed in cell culture. Note the neurite outgrowth is required for synapse formation to occur. Scale bar = 100 µm. Taken from Syed et al. (1990). **(B)** A pair of isolated *Lymnaea* neurons cultured in a soma-soma configuration. Using this approach, a synapse will form between somata in the absence of neurite outgrowth. Scale bar = 30 µm.

1.12 Main Objectives

The main aim of this thesis was to examine the role of RA and retinoid signaling in learning and memory formation using the mollusc *Lymnaea stagnalis.* As there is evidence that retinoid signaling is conserved between vertebrates and invertebrates I hypothesized that the role of RA in vertebrate memory formation may also be conserved in an invertebrate species. This research used both behavioural observations, as well as the examination of individual soma-soma synapses in cell culture to determine whether RA is involved in memory formation and/or synaptic plasticity in an invertebrate species.

The first aim of this research was to investigate whether retinoid signaling is required for successful learning and memory formation in *Lymnaea*. Retinoid signaling was impaired by exposing *Lymnaea* to either RALDH enzyme inhibitors or retinoid receptor antagonists, and both ITM and LTM formation were assessed. Additionally, retinoid receptor agonists were used to determine whether stimulating the retinoid signaling pathway could enhance memory formation.

Next, I aimed to examine whether RA itself would influence memory formation. However, RA is light-sensitive, and I discovered that exposure to constant darkness prior to operant conditioning enhances memory formation in *Lymnaea*. Based on this novel finding, my next aim was to combine retinoid signaling inhibition with the memory-enhancing effects of darkness to determine which effect would predominate. This study shed light on how environmental conditions, such as darkness, may interact with retinoid signaling to influence memory formation.

My third aim was to examine whether RA influences memory formation at the synaptic level, as synaptic plasticity is known to underlie memory formation. I used individual soma-

soma synapses in culture in order to examine whether RA influences synapse formation and/or synaptic plasticity as a means of understanding how RA is involved in memory formation.

Overall, this thesis investigates for the first time whether retinoid signaling is involved in memory formation in an invertebrate species.

Chapter 2:

Retinoid signaling is necessary for, and promotes long-term memory formation following operant conditioning

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2.01 Abstract

Retinoic acid, a metabolite of vitamin A, is proposed to play an important role in vertebrate learning and memory, as well as hippocampal-dependent synaptic plasticity. However, it has not yet been determined whether retinoic acid plays a similar role in learning and memory in invertebrates. In this study, we report that retinoid signaling in the mollusc *Lymnaea stagnalis*, is required for long-term memory formation following operant conditioning of its aerial respiratory behaviour. Animals were exposed to inhibitors of the RALDH enzyme (which synthesizes retinoic acid), or various retinoid receptor antagonists. Following exposure to these inhibitors, neither learning nor intermediate-term memory (lasting 2 hours) was affected, but long-term memory formation (tested at either 24 or 72 hours) was inhibited. We next demonstrated that various retinoid receptor agonists promoted long-term memory formation. Using a training paradigm shown only to produce intermediate-term memory (lasting 2 hours, but not 24 hours) we found that exposure of animals to synthetic retinoids promoted memory formation that lasted up to 30 hours. These findings suggest that the role of retinoids in memory formation is ancient in origin, and that retinoid signaling is also important for the formation of implicit memories, in addition to its previously demonstrated role in hippocampal-dependent memories.

2.02 Introduction

Retinoic acid, the active metabolite of vitamin A, is essential for vertebrate CNS development, playing an important role in patterning, differentiation (Maden, 2002, 2007) and neurite outgrowth (Corcoran et al., 2000; Dmetrichuk et al., 2005; Clagett-Dame et al., 2006). However, retinoic acid is also found in the adult brain (Dev et al., 1993), in areas such as the cortex, cerebellum and hippocampus (Kane et al., 2005). The hippocampus (including that of humans) also contains retinoid receptors (Krezel et al., 1999; Zetterström et al., 1999; Fragoso et al., 2012) and retinol-binding proteins (Zetterström et al., 1994). These discoveries led to the hypothesis that retinoid signaling may be important for hippocampal-dependent functions in the adult brain, such as learning and memory. Indeed, disruptions in retinoid signaling in the adult hippocampus can reduce hippocampal synaptic plasticity (Chiang et al., 1998; Misner et al., 2001) and impair spatial working memory (Chiang et al., 1998), novel object recognition (Wietrzych et al., 2005) and social recognition (Nomoto et al., 2012) in mice. Interestingly, there have been no studies reporting a role for retinoic acid in memory formation following operant or classical conditioning, particularly of an invertebrate species. In this study we investigated whether retinoic acid is required for learning and/or memory consolidation following operant conditioning of the aerial respiratory behaviour of the pond snail *Lymnaea stagnalis*. *Lymnaea* are conditioned by applying a punishing stimulus to the open pneumostome each time they perform aerial respiration and this results in a significant reduction in the behaviour (Lukowiak et al., 1996, 1998, 2000). The animals can form both intermediate-term memory (ITM; Lukowiak et al., 2000) and long-term memory (LTM; Lukowiak et al., 2000; Lowe and Spencer, 2006), depending on the number and length of training sessions given.

Endogenous retinoic acid is derived from vitamin A in two steps. First, vitamin A is oxidized into retinal via alcohol dehydrogenases. Retinal is then oxidized to retinoic acid via

retinaldehyde dehydrogenases (RALDH). Both reactions are required for the successful production of retinoic acid in many vertebrates. Alternative ancestral synthesis pathways have also been described, but most of these converge on retinal and thus still require the final oxidative reaction by RALDH (Simões-Costa et al., 2008). Retinoic acid generally acts by binding to nuclear retinoid receptors which then act as transcription factors to regulate gene expression. There are two classes of receptors in vertebrates: retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Mey and McCaffery, 2004). Though protostomes were thought to possess only RXRs, we recently cloned the first protostome RAR (*Lym*RAR; Genbank Accession no. GU932671) from the *Lymnaea* CNS. *Lym*RXR (Carter et al., 2010; Genbank Accession no. AY846875) and *Lym*RALDH (Genbank Accession no. FJ539101) have also been cloned. The *Lymnaea* CNS also contains retinoic acid and we have previously demonstrated RALDH activity in *Lymnaea* CNS extracts (Dmetrichuk et al., 2008). We have also shown that retinoic acid exerts similar regenerative effects on *Lymnaea* neurons as it does on vertebrate neurons (Dmetrichuk et al., 2006, 2008) and that retinoid receptor antagonists block, whilst synthetic retinoid agonists mimic, various effects of retinoic acid on *Lymnaea* neurons (Carter et al., 2010; Carter, 2011; Rand, 2012; Vesprini and Spencer, 2014).

In this study, we determined whether disruption of retinoid signaling in the mollusc *Lymnaea* would result in deficits in learning and/or memory following operant conditioning of the aerial respiratory behaviour. To this end, we used retinoid receptor antagonists as well as inhibitors of the RALDH enzyme. We found that following either of these treatments, long-term memory formation in *Lymnaea* (but not learning or intermediate-term memory formation) was inhibited. We also found that retinoid receptor agonists could promote long-term memory formation. These data suggest that retinoid signaling plays an important role in implicit memory formation in this molluscan species.

2.03 Materials and Methods

Animals

Lymnaea stagnalis (originally obtained from stocks at the Vrije University, Amsterdam) were bred in the laboratory environment. Adult snails were used for experimental procedures and ranged in shell length from 23 to 30 mm. These animals were maintained at room temperature on a 12 hour fixed light-dark cycle, in well-aerated artificial pond water (made with Instant Ocean salts; Aquarium Systems, Ohio, USA) and fed both NutraFin Max Spirulina fish food (Hagen) and romaine lettuce. *Lymnaea* were labeled with coloured markings on their shells at least 24 hours prior to the initiation of experimental procedures and were free to perform aerial respiration prior to any experimentation.

Chemicals

The RALDH inhibitors, citral and 4-diethylaminobenzaldehyde (DEAB), were obtained from Sigma-Aldrich. Citral was prepared in 70 % EtOH and diluted in artificial pond water to a final concentration of 50 µM citral (0.35 % EtOH). DEAB was prepared in 70 % EtOH and diluted in artificial pond water to a final concentration of 100 µM DEAB (0.35 % EtOH). Control trials were performed with 0.35 % EtOH (vehicle control).

The retinoid receptor antagonists were a generous gift from Dr. H. Kagechika (University of Tokyo, Japan). The RAR pan-antagonist LE540, the RARβ-selective antagonist LE135, and the RXR pan-antagonists PA452 and HX531 were prepared in 100 % DMSO and diluted in artificial pond water to a final concentration of 10^{-6} M. These concentrations of LE540, HX531 and PA452 have previously been shown to block various effects of retinoic acid on *Lymnaea* neurons (Carter et al., 2010; Carter, 2011; Rand, 2012, Vesprini and Spencer, 2014). Controls

were run to match the highest concentration of DMSO used in each experimental procedure (0.01 % for HX531 incubation, 0.05 % for LE540, LE135, and PA452 incubations, and 0.06 % when the combined effects of LE540 and PA452 were examined). The RXR pan-agonist PA024 was also a generous gift from Dr. H. Kagechika (University of Tokyo, Japan). The other retinoid receptor agonists, AM80, Ch55, and EC23, were obtained from Tocris Bioscience (Bristol, UK). All retinoid receptor agonists were prepared in 100 % DMSO and diluted in artificial pond water to a final concentration of 10^{-6} M. These agonists have been previously shown to mimic the effects of retinoic acid on *Lymnaea* neurons (Carter et al., 2010) or *Lymnaea* embryonic development (*unpublished observations*). Control trials were run with 0.01 % DMSO (vehicle control). All RALDH inhibitors and retinoid receptor agonists and antagonists were added to the bathing medium, consistent with previous studies (Rinkevich et al., 2007; Mathew et al., 2009; Carter et al., 2011).

Operant conditioning procedures

Operant conditioning of the aerial respiratory behaviour was performed as described previously (Lukowiak et al., 1996, 2000; Khan and Spencer, 2009). Snails were placed in a 1 L test beaker containing 800 mL of artificial pond water, bubbled with 100 % N_2 gas for 20 minutes prior to, and during all training sessions and memory tests. This hypoxic environment encouraged *Lymnaea* to perform aerial respiration instead of cutaneous respiration (Lukowiak et al., 1996). Animals were acclimatized to the hypoxic environment for 10 minutes prior to each training session or memory test (unless otherwise stated). *Lymnaea* were then gently propelled to the bottom of the test beaker to mark the start of each session. Training sessions were 45 minutes in duration with a 1 hour consolidation period between sessions. During each training session the conditioned animals received a tactile stimulus to the open pneumostome each time aerial

respiration was attempted. The strength of this stimulus was sufficient to induce pneumostome closure, but not sufficient to trigger the whole body withdrawal response. Yoked control animals received the same number of tactile stimuli as the operantly conditioned animals, though the application of a tactile stimulus to the yoked controls was not dependent upon pneumostome opening. Instead, a tactile stimulus was applied to an area proximal to the pneumostome each time the animal to which they were yoked attempted aerial respiration (Lukowiak et al., 1996, 1998). The number of attempts at aerial respiration during each session was recorded for each animal. Animals were then returned to the eumoxic home tank between training sessions, unless otherwise specified.

To produce LTM lasting 24 hours, animals received four 45 minute training sessions (S1 to S4) spaced 1 hour apart. First of all, we tested the animals' behaviour as a result of operant conditioning using "freely-behaving" test periods. In these experiments (Figure 6B and C only), the animals were allowed to freely perform aerial respiration in the pre-test, prior to training. The animals were then conditioned over four training sessions and given a freely-behaving post-test, 24 hours later. The number of observed pneumostome openings and total breathing time were then compared between the pre-test and post-test to determine whether a change in behaviour had occurred. For the remainder of the experiments, pre- and post-tests were not performed. Instead, LTM was tested at 24 hours by using memory tests in which the animals received stimuli during pneumostome openings. This protocol has previously been reported to be as effective in assessing changes in behaviour as the freely-behaving pre- and post-tests (Spencer et al., 2002; Lowe and Spencer, 2006). Previous studies using this training paradigm have also clearly shown that the reduction in behaviour as a result of learning and memory cannot be explained by either non-contingent tactile stimulation (Lukowiak et al., 1996, 1998, 2000) or by the hypoxic conditions (Lukowiak et al., 1996; Lowe and Spencer, 2006).

To produce LTM lasting 72 hours, animals received three 45 minute training sessions a day for three consecutive days (S1 to S9) with a 1 hour consolidation period between sessions on each day. A memory test was conducted 72 hours after the final training session (S9).

To produce intermediate-term memory (ITM) lasting 2 hours, the training procedure consisted of two 45 minute training sessions (S1 to S2) with a 1 hour consolidation period between sessions. The memory test was conducted either 2 hours after the final training session (to test for ITM), or 24 or 30 hours after the final training session (to test for LTM).

Procedures for chemical incubations

I) RALDH inhibitors:

To assess the role of retinoic acid in memory formation, two different inhibitors of the RALDH enzyme, citral and DEAB, were used. Animals were randomly assigned to three groups and incubated in either i) 500 mL of the RALDH inhibitor citral (50 μM; Marsh-Armstrong et al., 1994), ii) 500 mL of the RALDH inhibitor 4-diethylaminobenzaldehyde (DEAB; 100 μM; Rinkevich et al., 2007; Le et al., 2012), or iii) 500 mL of vehicle (0.35 % EtOH) as a control. The inhibitor concentrations used in this study were similar to those used in vertebrate studies and their effectiveness and specificity are well documented (Russo et al., 1988; Kikonyogo et al., 1999; Koppaka et al., 2012). A 72 hour incubation time in the RALDH inhibitors was used, based on positive results from previous research using similar (or lower) incubation times (Prince and Carlone, 2003; Mathew et al., 2009). In some experiments only citral was used, as all other experiments produced similar results with both citral and DEAB.

For the experiments investigating ITM and 24 hour LTM, all animals were incubated in the RALDH inhibitor (or vehicle) for 72 hours prior to the first training session (S1) in 1 L

beakers, open to the surrounding environment and bubbled with air. Animals were removed from these beakers at the start of S1 and placed in eumoxic home tanks containing artificial pond water between all training sessions and until the memory test. The acclimatization period prior to S1 was extended from 10 minutes to 30 minutes to give the animals additional time to acclimatize to the change in solutions (from inhibitor to pond water). All animals were operantly conditioned and the number of attempts at aerial respiration was recorded for all animals during the training sessions and memory test for analysis. In the 72 hour LTM experiments, the inhibitor incubations were conducted at different points during the training procedure. *Lymnaea* were either incubated for a) 72 hours beginning at the first training session (S1) and ending at S9, or b) 72 hours immediately following the final training session (S9) and ending at the memory test.

II) Retinoid Receptor Antagonists:

In these experiments, animals were conditioned either to produce 24 hour LTM or 2 hour ITM. All animals were incubated in the antagonists in aerated 400 mL beakers, starting 24 hours before the first training session (S1), ensuring sufficient time to inhibit retinoid signaling prior to the start of training. Incubations were maintained throughout training and until the memory test.

Animals were only removed from the 400 mL incubation beakers during the training sessions and memory test. Animals were incubated in 200 mL of either i) the RXR pan-antagonist HX531 (10^{-6} M), ii) the RXR pan-antagonist PA452 (10^{-6} M), iii) the RAR pan-antagonist LE540 (10^{-6} M), iv) the RARβ-selective antagonist LE135 (10^{-6} M), v) a combination of PA452 and LE540 (10^{-6} M), or vi) DMSO (vehicle control; 0.01 % to control for HX531; 0.05 % to control for incubation in LE540, LE135, and PA452; 0.06 % to control for the combination of antagonists).

III) Retinoid Receptor Agonists:

All animals were incubated in the agonists in aerated 400 mL beakers beginning 24 hours before the first training session (S1), and incubations were maintained until the memory test. Animals were only removed from the incubation beakers during the training sessions and memory test and were immediately returned to the agonist solutions following completion of a training session. Animals were operantly conditioned with a training procedure that normally produces ITM (i.e. memory lasting 2 hours) and a memory test was administered 30 hours after the final training session to test for LTM formation. Animals were incubated in 200 mL of either i) DMSO (0.01 %; vehicle control), ii) the RARα/β agonist Ch55 (10^{-6} M), iii) the RXR pan-agonist PA024 (10^{-6} M), iv) the RARα agonist AM80 (10^{-6} M), or v) the synthetic retinoid EC23 (10^{-6} M). The Am- and Ch-series of benzoic acid derivatives elicit the same cellular responses as retinoic acid in several cell systems (Jetten et al., 1987; Sato et al., 1988) and EC23 mimics the effects of all-trans RA (Clemens et al., 2013) showing competitive binding toward vertebrate RARs, but not RXRs (Christie et al., 2008).

Data and statistical analysis

Learning was operationally defined in this study as a significant reduction in the number of attempted pneumostome openings from the first training session to the final training session (Lukowiak et al., 1996), compared to yoked controls. Memory was deemed to have occurred when a) the number of attempted pneumostome openings in the memory test was significantly lower than the first training session but not significantly different from the final training session (Lukowiak et al., 1998), and when b) the number of openings in the memory test of conditioned animals was significantly less than the number of openings in the memory test of yoked controls.

(In the case of the freely-behaving memory tests, memory was defined as a significant decline in the number of pneumostome openings and total breathing time in the post-test compared to the pre-test). All data are presented as mean ± SEM unless otherwise stated. Data were square root transformed prior to statistical analysis when data did not pass the equal variance test. We used a mixed factor ANOVA, where drug condition was specified as the fixed factor and session was specified as the random, repeated factor. A Tukey *post-hoc* test was used for comparisons, which were considered to be significant when $p < 0.05$.

2.04 Results

Operant conditioning of aerial respiratory behaviour

In this study, we first used a conditioning procedure similar to that used in Lowe and Spencer (2006), consisting of four 45 minute training sessions (S1 to S4) separated by 1 hour, in order to generate LTM at 24 hours. A 45 minute memory test (MT) was administered 24 hours after the final training session (S4). A mixed factor ANOVA on conditioned and yoked control data revealed a significant interaction effect between condition and session ($F_{(2,56)} = 15.48$; $p < 0.001$). *Post-hoc* analysis indicated that this procedure resulted in learning (i.e. a significant decrease in attempts at aerial respiration from S1 (9.80 ± 0.74) to S4 (4.33 ± 0.64; $p < 0.001$)) and LTM formation (MT: 4.87 ± 0.52; $p < 0.001$) in the operantly conditioned animals ($n = 15$; Figure 6Ai). Yoked controls ($n = 15$) did not show either learning or LTM formation (S1: 8.07 ± 0.69; S4: 7.80 ± 0.60; MT: 8.60 ± 0.83; $p > 0.05$; Figure 6Aii).

As in previous studies (Lukowiak et al., 1996; Lowe and Spencer, 2006), we also used freely behaving pre- and post-test sessions to confirm that aerial respiratory behaviour was indeed reduced in the operantly conditioned group. Using a separate cohort of animals from those used above, all animals were given a 45 minute pre-test before the first training session (S1) and a 45 minute post-test 24 hours after the last training session (S4), during which they were permitted to freely perform aerial respiration (i.e. freely-breathing MT). The mixed factor ANOVA analysis revealed a significant interaction between condition and session for both the number of pneumostome openings ($F_{(1,36)} = 16.62$; $p < 0.001$) as well as total breathing time ($F_{(1,36)} = 27.00$; $p < 0.001$). *Post-hoc* analysis showed that, as in the previous paradigm, only operantly conditioned animals showed learning and a reduction in the number of openings from S1 (8.58 ± 0.57) to S4 (4.63 ± 0.43; $p < 0.001$; $n = 19$; data not shown). Yoked control animals ($n = 19$) showed no change in the number of observed pneumostome openings from S1 ($7.79 \pm$

0.58) to S4 (7.53 ± 0.71; $p > 0.05$; data not shown). The operantly conditioned animals showed significantly fewer pneumostome openings during the freely-breathing MT compared to the number of openings in the pre-test ($p < 0.001$; Figure 6Bi), whereas yoked control animals showed no significant change in the number of pneumostome openings in the post-test compared to the pre-test ($p > 0.05$; Figure 6Bii). The operantly conditioned animals also spent significantly less time performing aerial respiration during the freely-breathing MT compared to the pre-test ($p < 0.001$; Figure 6Ci) whereas the yoked controls showed no significant change in the amount of time spent performing aerial respiration during the pre-test and MT sessions ($p > 0.05$; Figure 6Cii). These experiments demonstrate a reduction in respiratory behaviour in conditioned animals, but no change in behaviour in the yoked control groups, and confirm what has been shown in previous studies in *Lymnaea* (Lukowiak et al., 1996; Lowe and Spencer, 2006; Khan and Spencer, 2009), that learning and memory result only from contingent stimulation of the open pneumostome.

RALDH inhibitors perturbed LTM formation

In order to examine any potential involvement of retinoic acid in learning and memory formation, *Lymnaea* were first incubated in the RALDH inhibitors citral (50 μM), DEAB (100 μM), or 0.35 % EtOH (vehicle control) for 72 hours prior to S1. Animals in all three groups were operantly conditioned with the same procedure used above, that normally generates LTM 24 hours later, and a mixed factor ANOVA revealed a significant interaction effect between condition and session ($F_{(4,82)} = 7.40$: $p < 0.001$). *Post-hoc* analysis indicated that incubation in EtOH did not result in any significant impairment in either learning or LTM formation.

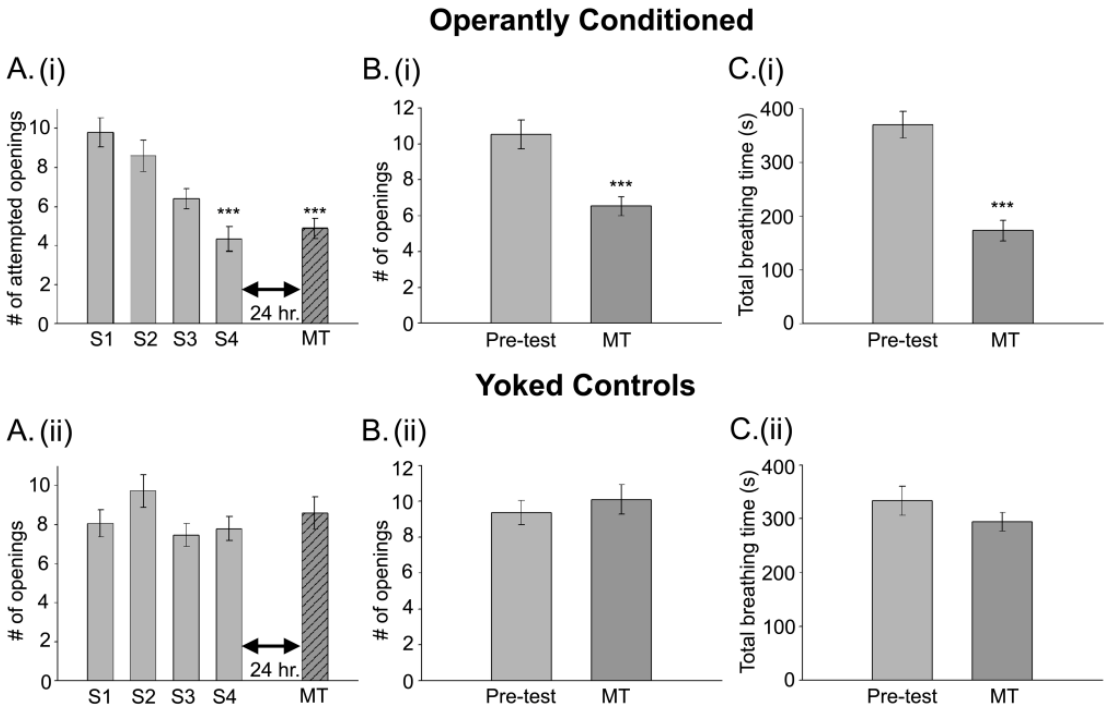

Figure 6. Four training sessions produce LTM lasting 24 hours. (A) All animals received four 45 minute training sessions (S1-S4) spaced 1 hour apart, followed by a 45 minute MT, 24 hours after the final training session (S4). **(i)** Operantly conditioned animals (n = 15) demonstrated learning and LTM formation (*** $p < 0.001$ relative to S1). **(ii)** Yoked controls (n = 15) showed no change in aerial respiratory behaviour across training sessions (S1-S4) or in the MT ($p > 0.05$ relative to S1). The behaviour in S4 and the MT were significantly different between conditioned and yoked control groups ($p < 0.001$). **(B)** and **(C)** A separate cohort of animals were given four training sessions as in **A.** but were given a 45 minute freely-behaving pre-test before S1 and a 45 minute freely-behaving MT (post-test) 24 hours after the final training session (S4). **(Bi)** Operantly conditioned animals (n = 19) made significantly fewer attempts at aerial respiration during the freely behaving MT relative to the freely-behaving pre-test session (*** $p < 0.001$). **(Bii)** Yoked control animals (n = 19) showed no significant difference in the number of pneumostome openings in the freely-behaving MT relative to the pre-test session. **(Ci)** Operantly conditioned animals (n = 19) also demonstrated a significantly lower total breathing time during the freely-behaving MT relative to the pre-test session (*** $p < 0.001$). **(Cii)** Yoked controls (n = 19) showed no significant difference in total breathing time in the freely-behaving MT relative to the pre-test session.

As expected, these animals showed a significant decrease ($p < 0.001$) in attempts at aerial respiration from S1 to S4, indicative of successful learning, as well as a significant reduction in behaviour between S1 and the MT ($p < 0.001$; $n = 15$), showing LTM formation (Figure 7A). Incubation in either of the RALDH inhibitors did not affect learning, but did impair LTM formation. Animals incubated in 50 μM citral for 72 hours prior to training ($n = 15$) showed learning, observed as a significant decrease in attempts at respiration from S1 to S4 ($p < 0.001$). However, this reduction in behaviour was not maintained for 24 hours, as these animals attempted aerial respiration significantly more often in the MT compared to S4 ($p < 0.001$; Figure 7B). Animals incubated in 100 μM DEAB ($n = 14$) also showed learning (S1 to S4: $p < 0.001$); however, this reduction in behaviour was not maintained for 24 hours, as these animals also attempted aerial respiration significantly more often in the MT compared to S4 ($p < 0.001$; Figure 7C). Figure 7D illustrates the change in behaviour in the MT (normalized to S1) for each group and demonstrates that the behaviour in both citral and DEAB was not significantly different from that of yoked controls, but was significantly different from the trained animals incubated in EtOH ($p < 0.05$). These data suggest that retinoid signaling may be required for successful LTM formation, as assessed 24 hours after training.

RALDH inhibitors also impaired LTM at 72 hours

The data shown above suggest that incubating the animals in RALDH inhibitors for 72 hours prior to training prevented LTM formation. We next investigated the role of retinoic acid at different time points during operant conditioning and designed a new training procedure to apply the RALDH inhibitor for 72 hours at different stages of the training procedure. Training sessions now spanned 72 hours, with 3 training sessions per day for a total of 9 training sessions

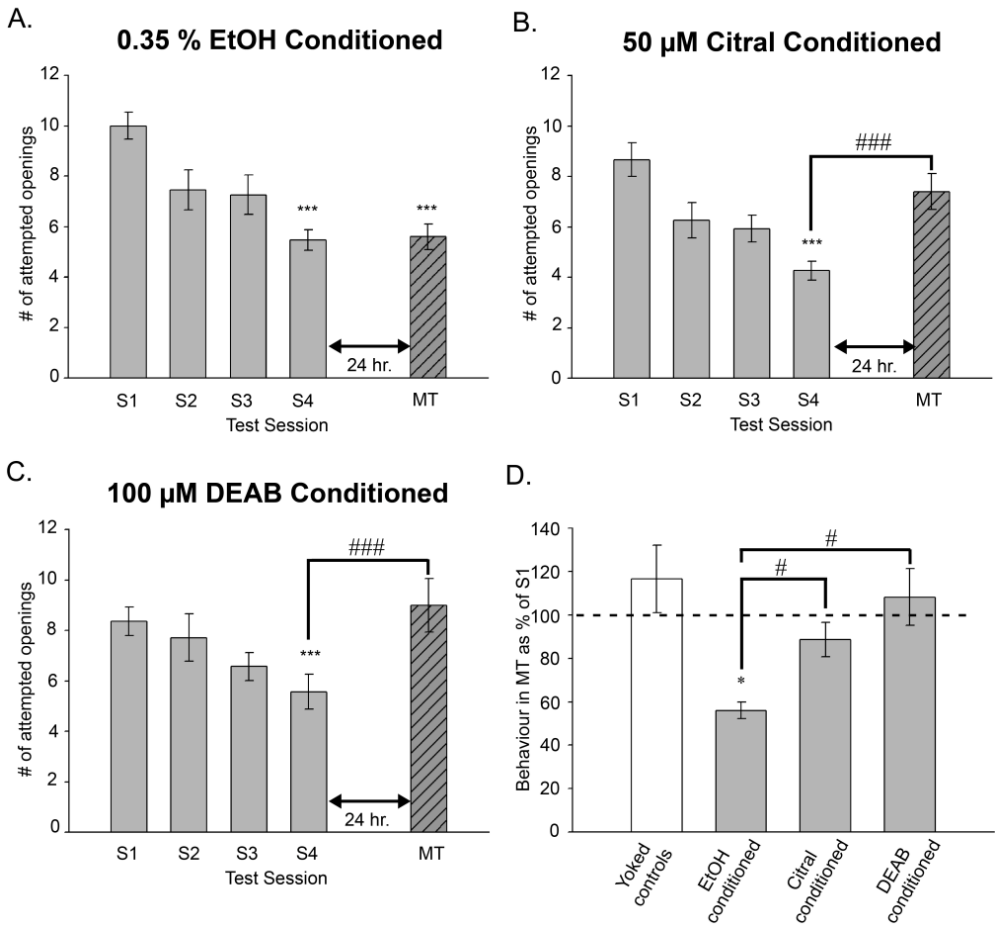

Figure 7. LTM formation is inhibited following incubation in RALDH inhibitors. Animals were incubated in various conditions for 72 hours before S1. All animals were then operantly conditioned using four 45 minute training sessions, followed by a MT 24 hours later. **(A)** Animals incubated in 0.35 % EtOH (n = 15) demonstrated both learning and LTM (*** $p < 0.001$ relative to S1). **(B)** and **(C)** Animals incubated in the RALDH inhibitors **B**: citral (n = 15) and **C**: DEAB (n = 14) demonstrated learning (*** $p < 0.001$ relative to S1). However, LTM formation was impaired, as these animals attempted aerial respiration significantly more often in the MT than in S4 (### $p < 0.001$). Furthermore, the number of pneumostome openings in the MT was not significantly different from that observed in S1 ($p > 0.05$). **(D)** Summary showing the behaviour exhibited in the MT of each group (normalized to S1). Only the conditioned vehicle group (EtOH) demonstrated a significant difference from yoked controls (* $p < 0.05$), and both conditioned groups in citral and DEAB showed a significant difference from the conditioned group in EtOH (# $p < 0.05$; Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunn's *post-hoc* performed on data in **D** only).

(S1 to S9). The MT was conducted 72 hours after the last training session (Figure 8Ai). This allowed us to incubate animals in the RALDH inhibitor for a) 72 hours *during* training or b) 72 hours *following* training, but prior to the MT. We first showed that operantly conditioned animals (n = 12) showed both learning and memory lasting 72 hours with this procedure (Figure 8Aii), whereas yoked control animals (n = 12) did not show any significant change in behaviour (Figure 8Aiii). A mixed factor ANOVA revealed a significant interaction effect ($F_{(2,44)} = 12.91$; $p < 0.001$), with a significant reduction in openings from S1 to S9 and from S1 to MT in operantly conditioned animals only. Yoked controls showed no significant differences between S1 and S9 or the MT.

Animals were next incubated in either citral or EtOH (vehicle) for the 72 hour period beginning at S1 (Figure 8Bi), and both groups were conditioned using the 72 hour training paradigm. A mixed factor ANOVA revealed a significant interaction effect ($F_{(2,56)} = 7.28$; $p = 0.002$). Animals incubated in EtOH (0.35 %; n = 15) demonstrated both learning and memory ($p < 0.001$; Figure 8Bii). The animals incubated in citral (50 µM; n = 15) displayed successful learning ($p < 0.001$), but these animals showed a significant increase in aerial respiratory activity in the MT relative to their last training session ($p < 0.01$) as well as a significant increase in behaviour compared to the MT of the conditioned EtOH group ($p < 0.01$). However, the behaviour of the citral-treated animals did not rebound completely, as the number of openings in the MT remained less than that initially observed in S1 ($p < 0.001$; Figure 8Biii).

A mixed factor ANOVA on data collected from animals incubated for the 72 hour period immediately after the completion of the last training session (S9; Figure 8Ci) revealed a significant within-group difference only ($F_{(2,56)} = 102.92$; $p < 0.001$). Animals incubated in EtOH (0.35 %; n = 15) again demonstrated both learning and memory (Figure 8Cii). The animals incubated in citral (50 µM; n = 15) also demonstrated successful learning ($p < 0.001$). However,

unlike the previous group, these animals also successfully formed LTM lasting 72 hours, as there was no significant difference between S9 and the MT ($p > 0.05$) and the number of attempted pneumostome openings in the MT was significantly less than that of S1 ($p < 0.001$; Figure 8Ciii).

These data demonstrated that animals incubated in citral for 72 hours during the training procedure showed an increase in behaviour in the MT, showing impaired LTM. However, animals incubated in the RALDH inhibitor once training was complete, showed no inhibition of LTM formation. Together, the above data suggest that retinoid signaling during training is likely required for successful LTM formation.

Retinoid receptor antagonists impair LTM formation

Retinoic acid can regulate gene transcription by binding to both retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Mey and McCaffery, 2004; Marlétaz et al., 2006), both of which have been cloned from *Lymnaea* (Carter et al., 2010; Carter, 2011). Our next aim was thus to determine whether blocking the retinoid receptors produced the same effect on LTM formation as inhibition of RALDH activity. Animals were incubated in either DMSO or various receptor antagonists: HX531, PA452 and LE540 (at 10^{-6} M), all of which have been previously shown to inhibit various effects of retinoic acid on *Lymnaea* neurons (Carter et al., 2010, Carter, 2011; Rand, 2012; Vesprini and Spencer, 2014). Incubations were carried out for 24 hours before the first training session (S1) and were maintained until the memory test, 24 hours after the last training session.

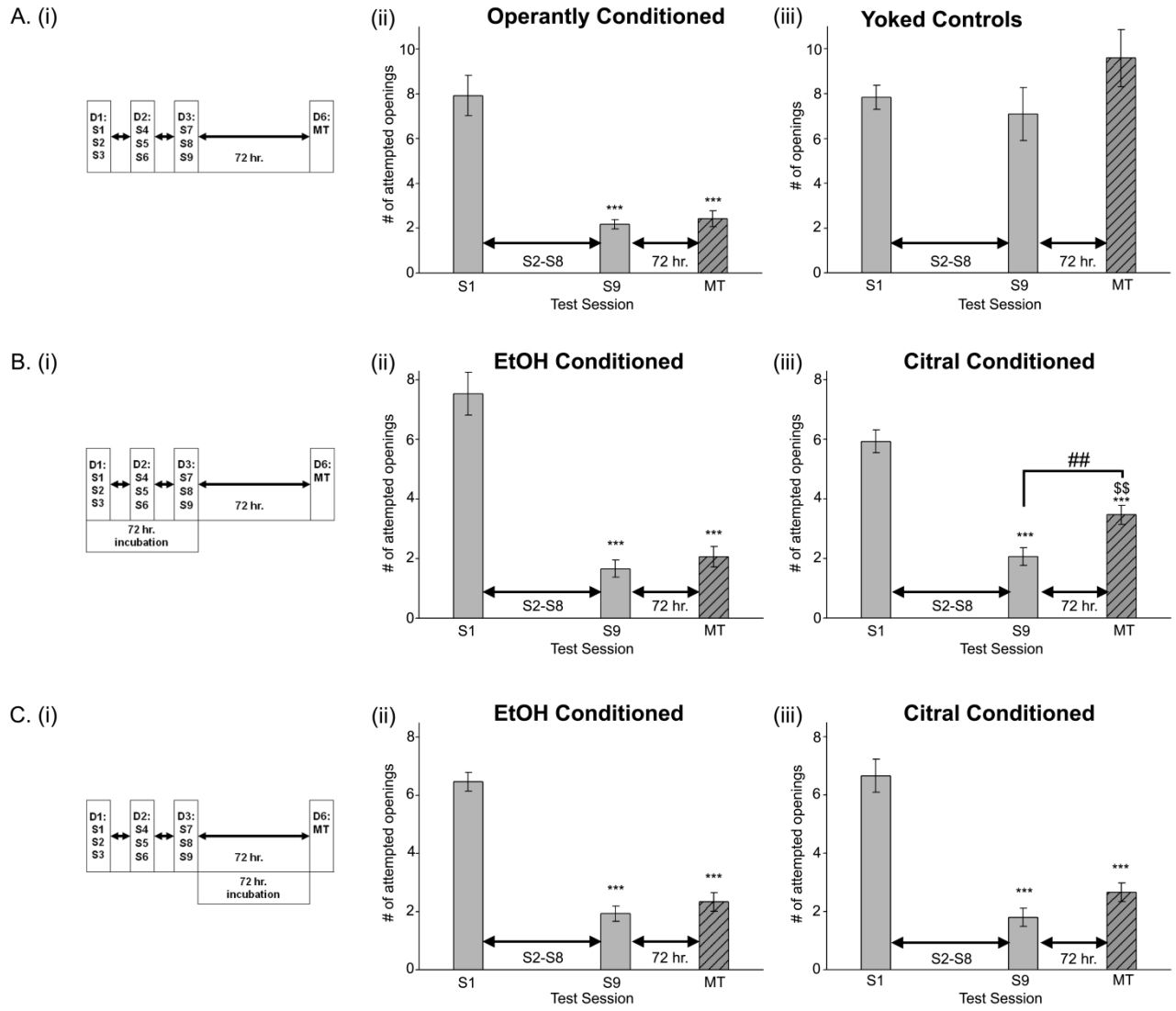

Figure 8. RALDH inhibition after training does not impair LTM formation. A 72 hour training protocol was used (S1 to S9), with a MT 72 hours after the last training session. Only the operantly conditioned group (n = 12; **Aii**), but not yoked controls (n = 12; **Aiii**), showed a significant change in their behaviour (*** $p < 0.001$ relative to S1). Animals were next incubated in either EtOH or citral for 72 hours during training **(Bi)** or after training **(Ci)** and both groups were conditioned. **(Bii)** Animals incubated in the vehicle (0.35 % EtOH) during training (n = 15) demonstrated learning and LTM formation **(Biii)** Animals incubated in citral (50 µM; n = 15) also demonstrated learning (*** $p < 0.001$ relative to S1), though these animals increased their aerial respiratory activity in the MT relative to S9, suggesting impairment of LTM formation (## $p < 0.01$). The behaviour in the MT of the citral group was also significantly different from that of the EtOH controls ($$ $p < 0.01$). **(Cii)** Animals incubated in the vehicle (0.35 % EtOH) for the 72 hour period immediately following the completion of training at S9 (n = 15) demonstrated learning and LTM formation. **(Ciii)** Animals incubated in citral (n = 15) also demonstrated both learning and LTM formation. *** $p < 0.001$ relative to S1.

As we have previously shown that HX531 is most effective in inhibiting both morphological and electrophysiological effects of retinoic acid on *Lymnaea* neurons (Carter et al., 2010; Vesprini and Spencer, 2014), we began our experiments with this antagonist. Yoked control animals incubated in HX531 (10^{-6} M; n = 9) showed no significant difference in their behaviour across any of the sessions (S1: 7.00 ± 0.55; S4: 6.67 ± 0.60) or memory test (MT: 6.67 ± 0.44; $p > 0.05$; Friedman Repeated Measures Analysis of Variance on Ranks; data not shown), confirming that the pharmacological treatment alone, had no significant effect on aerial respiratory behaviour. A separate group of animals were next incubated in either HX531 (10^{-6} M; n = 18) or DMSO as the vehicle control (0.01%; n = 15) and both groups were operantly conditioned using the same training protocol, with a MT 24 hours later. A mixed factor ANOVA revealed a significant interaction effect ($F_{(2,62)} = 5.68$; $p = 0.005$). Animals incubated in HX531 showed learning, with a significant reduction in pneumostome openings from S1 to S4 ($p < 0.001$; Figure 9A). However, these animals showed a complete impairment of LTM formation, as the number of attempted pneumostome openings in the MT was significantly greater than observed in S4 ($p < 0.001$) and significantly greater than the behaviour expressed by the conditioned vehicle controls ($p < 0.001$). DMSO controls (0.01 %; n = 15) demonstrated both learning and LTM ($p < 0.001$) as expected (S1: 8.40 ± 0.74; S4: 3.67 ± 0.71; MT: 4.33 ± 0.35; data not shown).

We next tested the pan-antagonists PA452 (RXR) and LE540 (RAR), as well as the RARβ-selective antagonist LE135. A mixed factor ANOVA revealed a significant within-group difference ($F_{(2,126)} = 59.85$; $p < 0.001$). Animals that were incubated in DMSO (0.05%; n = 16) again showed both learning and LTM formation 24 hours after training (Figure 9B). Animals incubated in either the RXR pan-antagonist PA452 (10^{-6} M; n = 18; Figure 9C) or the RAR pan-antagonist LE540 (10^{-6} M; n = 16; Figure 9D) demonstrated learning ($p < 0.001$), but the

treatment resulted in partial perturbation of LTM formation, as there was a significant increase in the number of openings from S4 to the MT in both conditions ($p < 0.05$). However, the number of openings in the MT remained significantly less than those observed in S1 (PA452: $p < 0.05$; LE540: $p < 0.01$). Interestingly, the RARβ-selective antagonist LE135 (10^{-6} M; n = 17) had no significant effect on either learning or LTM formation (Figure 9E).

The data above suggest that LE540 and PA452 had a partial effect on the behaviour in the MT, so we next aimed to determine whether treatment with a combination of these RAR and RXR pan-antagonists might have a synergistic effect to impair LTM formation in *Lymnaea*. Animals were incubated in either DMSO (0.06 %; n = 16) or a combination of LE540 and PA452 (10^{-6} M; n = 14) and all animals were conditioned as before. A mixed factor ANOVA revealed a significant interaction effect ($F_{(2,56)} = 3.84$; $p < 0.05$). Animals incubated in DMSO again showed both learning and memory as expected (S1: 8.81 ± 0.52; S4: 3.75 ± 0.52; MT: 4.63 ± 0.48; $p < 0.001$; data not shown). Animals exposed to the combination of LE540 and PA452 showed learning ($p < 0.001$), but did not show successful LTM consolidation at 24 hours, as the number of attempted pneumostome openings observed in the MT was significantly greater than that in S4 ($p < 0.01$; Figure 9F) and significantly greater than the DMSO controls ($p < 0.05$). Thus, incubation in both retinoid receptor antagonists together appeared to have a synergistic effect to inhibit LTM in *Lymnaea*.

These data demonstrate that inhibition of retinoid signaling by blocking retinoid receptors (using both RXR and RAR antagonists) had a similar effect in inhibiting LTM formation as blocking the activity of RALDH. These data further support a role for retinoid signaling in LTM formation.

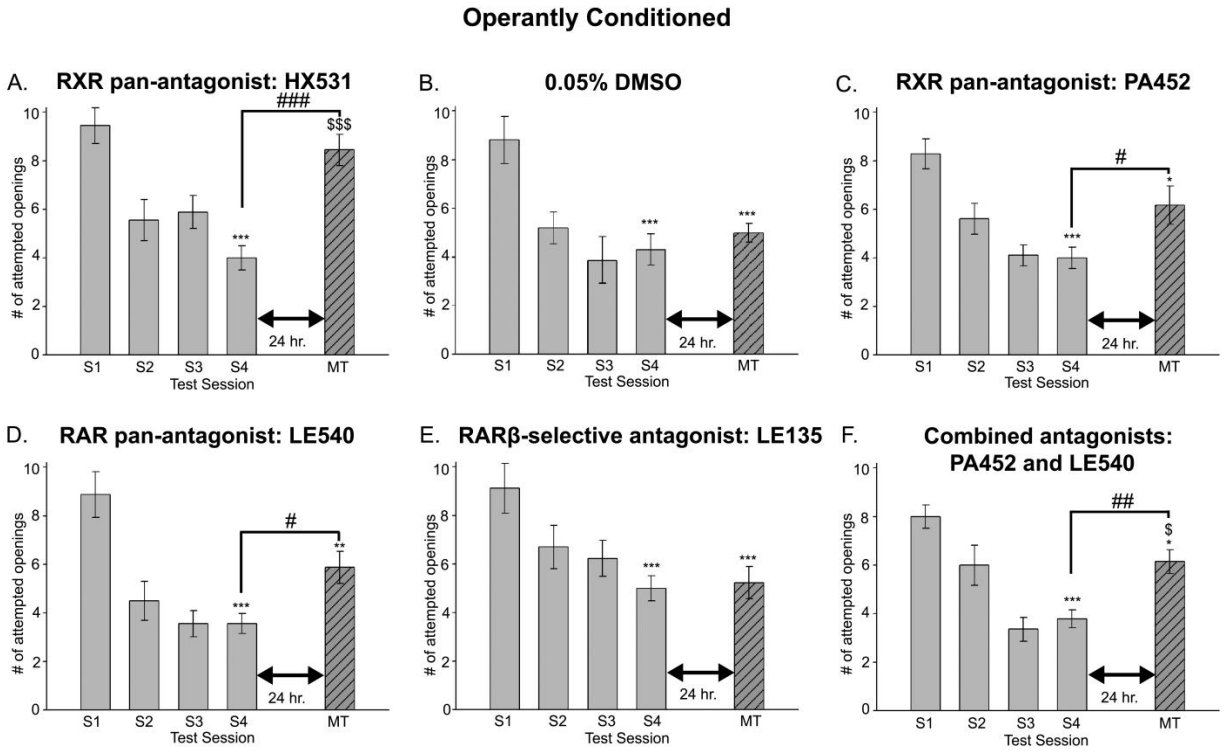

Figure 9. RAR and RXR pan-antagonists impair LTM formation. All animals were incubated in either the vehicle (DMSO) or retinoid receptor antagonists for a period beginning 24 hours before S1 and lasting until the MT. All groups were operantly conditioned using four 45 minute training sessions to produce LTM lasting 24 hours. **(A)** Animals incubated in the RXR pan-antagonist HX531 (10^{-6} M; n = 18) demonstrated learning and a complete impairment of LTM formation at 24 hours (*** $p < 0.001$, relative to S1; ### $p < 0.001$ relative to S4; $$$ $p < 0.001$ relative to MT of DMSO controls). **(B)** Animals incubated in 0.05 % DMSO (vehicle control; n = 16) demonstrated both learning and LTM formation (*** $p < 0.001$ relative to S1). **(C)** Animals incubated in the RXR pan-antagonist PA452 (10^{-6} M; n = 18) also demonstrated learning. However, aerial respiratory behaviour significantly increased during the MT, relative to S4, suggesting LTM impairment (# $p < 0.05$). **(D)** Animals incubated in the RAR pan-antagonist LE540 (10^{-6} M; n = 16) also demonstrated learning, but partial LTM impairment (# $p < 0.05$). **(E)** Animals incubated in the RARβ-selective antagonist LE135 (10^{-6} M; n = 17) demonstrated learning and LTM formation at 24 hours. **(F)** Animals incubated in the combination of RXR and RAR pan-antagonists (PA452 and LE540; 10^{-6} M; n = 14) also demonstrated learning. These animals significantly increased their attempts at aerial respiration in the MT relative to S4, suggesting LTM impairment (## $p < 0.01$). The behaviour in the MT was also significantly different from that of the conditioned group in DMSO (0.06 %; $ $p < 0.05$). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ relative to S1.

Inhibition of retinoid signaling does not affect intermediate-term memory formation

Retinoic acid is well-known to act as a transcriptional activator, but numerous studies have also implicated non-genomic effects of retinoic acid and signaling via its receptors. These include changes in transmitter release (Liao et al., 2004), control of protein synthesis (Poon and Chen, 2008) and dendritic spine formation (Chen and Napoli, 2008), all of which may lead to changes in synaptic strength and thus synaptic plasticity affecting memory formation. We have also shown non-genomic actions of retinoic acid in *Lymnaea* neurons (Farrar et al., 2009), as well as localization of both RXR (Carter et al., 2010) and RAR (Carter, 2011) to neurite processes. Our next aim was thus to investigate whether retinoic acid's effects on memory formation involves transcriptional activation. In *Lymnaea* it has previously been shown that successful LTM formation requires gene transcription, but intermediate-term (ITM) memory formation does not (Sangha et al., 2003a). Thus, in order to determine whether retinoic acid may be acting through its classical role in gene transcription, we investigated whether blocking either RALDH activity or receptor activation would also impair ITM formation.

Animals were subjected to an ITM training procedure (or a yoked control procedure), consisting of two 45 minute training sessions, spaced 1 hour apart, followed by a MT 2 hours later. The animals operantly conditioned with this procedure showed a significant decrease in attempted aerial respiration in S2 relative to S1 ($p < 0.001$). Following this training, memory was retained for 2 hours (S1 to MT: $p < 0.001$; $n = 14$; Figure 10Aii), but not for 24 hours (S1 to MT: $p > 0.05$; $n = 16$; Figure 10Bii). Yoked controls showed no significant change in aerial respiratory behaviour across the two training sessions or in the MTs at 2 hours ($n = 14$; Figure 10Aiii) and 24 hours ($n = 16$; Figure 10Biii) ($p > 0.05$). Thus, this training procedure was sufficient to produce ITM lasting 2 hours, but not LTM lasting 24 hours.

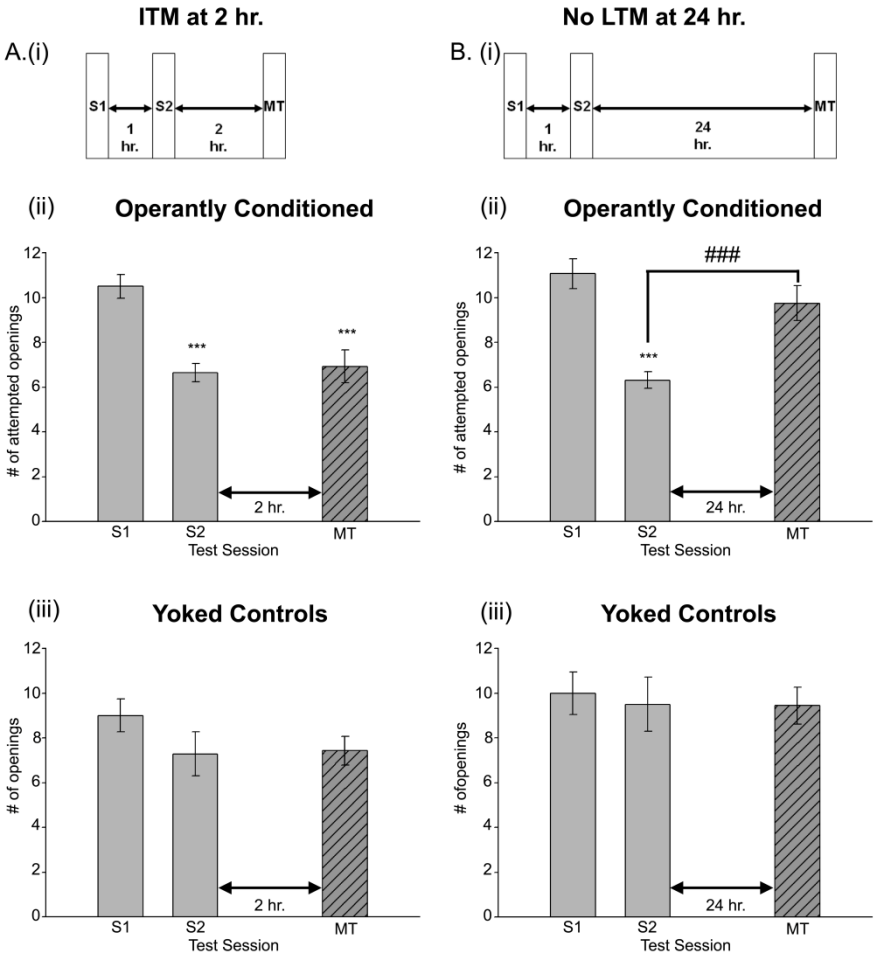

Figure 10. Two training sessions produce ITM at 2 hours, but not LTM at 24 hours. All animals were operantly conditioned with two 45 minute training sessions spaced 1 hour apart. One group of animals received the MT at 2 hours **(Ai)**, the other at 24 hours **(Bi)**. Operantly conditioned animals demonstrated learning, with a significant reduction in aerial respiratory activity from S1 to S2 (**Aii**. n = 14; **Bii**. n = 16). Operantly conditioned animals formed ITM lasting 2 hours **(Aii)**, but did not demonstrate LTM at 24 hours **(Bii)**. Yoked controls showed no significant change in aerial respiratory behaviour across training sessions or in the MTs (**Aiii**. n = 14; **Biii.** n = 16). *** $p < 0.001$ relative to S1; ### $p < 0.001$ relative to S2.

A different group of animals were next incubated in either citral (50 µM; n = 14), DEAB (100 µM; n = 13), or 0.35 % EtOH (n = 14), for 72 hours prior to the initiation of training (Figure 11A). All animals were operantly conditioned with the ITM procedure, and the number of attempted pneumostome openings was recorded for analysis. A mixed factor ANOVA revealed a significant within-group difference ($F_{(2,76)} = 42.54$; $p < 0.001$), but no significant between-group difference ($F_{(2,76)} = 0.75$; $p > 0.05$). That is, vehicle controls (0.35 % EtOH) showed both learning ($p < 0.001$) and ITM formation at 2 hours ($p < 0.001$ MT relative to S1; Figure 11B). Animals treated with citral (Figure 11C; $p < 0.01$) or DEAB (S1: 9.46 ± 0.64 to S2: 7.31 ± 0.63: $p < 0.05$; data not shown) also showed a significant decrease in aerial respiratory behaviour from S1 to S2, demonstrating successful learning. Animals exposed to either citral (Figure 11C; $p < 0.001$) or DEAB (S1 to MT (6.15 ± 0.76): $p < 0.001$; data not shown) also showed ITM formation at 2 hours. Thus, exposure to the RALDH inhibitors did not impair ITM formation following operant conditioning in *Lymnaea*, suggesting that RALDH enzymatic activity does not appear to be required for successful ITM consolidation.

As the most successful LTM inhibition was obtained with the RXR pan-antagonist HX531, we next tested the effect of this inhibitor on ITM formation. Animals were incubated in either DMSO (0.01 %) or HX531 (10^{-6} M) for 24 hours before the first training session, and the incubation was maintained until the MT (Figure 11D). A mixed factor ANOVA again revealed only a within-group difference ($F_{(2,74)} = 67.60$; $p < 0.001$). As shown previously, animals incubated in DMSO (n = 22) demonstrated both learning and ITM lasting 2 hours ($p < 0.001$; Figure 11E). However, animals incubated in HX531 (n = 22) also demonstrated both learning and ITM lasting 2 hours ($p < 0.001$; Figure 11F). Thus, exposure to the RXR pan-antagonist, HX531, did not impair the ability of *Lymnaea* to form ITM. Together, these findings suggest that

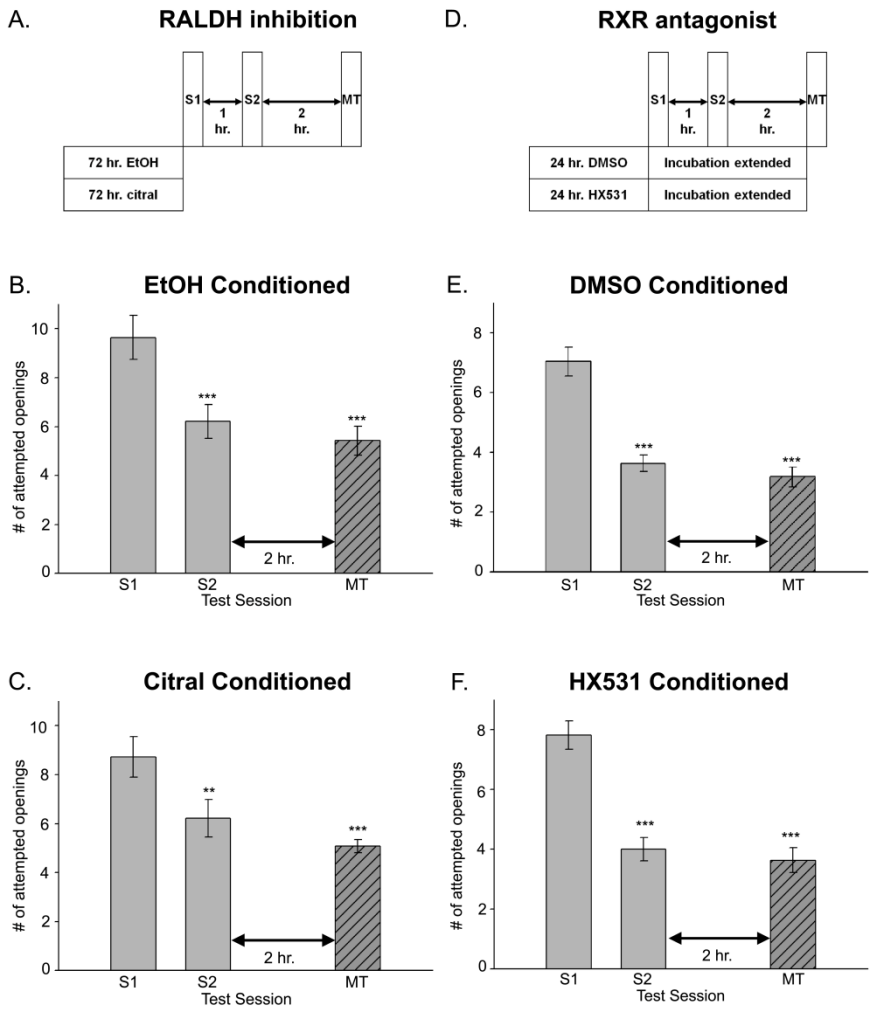

Figure 11. Inhibition of retinoid signaling does not prevent ITM formation. All animals were operantly conditioned using two 45 minute training sessions to produce ITM lasting 2 hours. **(A)** Animals were incubated for 72 hours before training, in either the vehicle control (0.35 % EtOH) or in citral (50 µM). **(B)** Animals incubated in EtOH (n = 14) demonstrated both learning and ITM formation at 2 hours. **(C)** Animals incubated in citral (n = 14) also demonstrated both learning and successful ITM formation at 2 hours. **(D)** Animals were incubated for 24 hours before training and until the MT, in either the vehicle (0.01 % DMSO) or the RXR pan-antagonist HX531 (10^{-6} M). **(E)** Animals incubated in DMSO (n = 22) demonstrated both learning and ITM formation at 2 hours. **(F)** Animals incubated in the RXR pan-antagonist HX531 (n = 22) also demonstrated both learning and ITM formation at 2 hours. *** $p < 0.001$, ** $p < 0.01$ relative to S1.

retinoic acid signaling is not required for ITM formation and thus support the notion that it may be required for transcriptional activation during LTM formation.

Retinoid receptor agonists promote LTM formation

The data shown above suggest that retinoid signaling via its receptors, is required for LTM formation (but not ITM) in *Lymnaea* following operant conditioning. We next hypothesized that activation of retinoid receptors, using synthetic receptor agonists, may actually promote long-term memory formation in *Lymnaea*. To test this hypothesis, we incubated animals in various retinoid receptor agonists for 24 hours prior to training and until the MT. We then used the ITM training procedure consisting of two training sessions (shown previously not to produce LTM at 24 hours; Figure 10). In these experiments, we even extended the duration from the last training session and tested for LTM formation after 30 hours. Animals were treated with 10^{-6} M agonists Ch55 (RARα/β; n = 16); PA024 (pan-RXR; n = 15), AM80 (RARα; n = 16), the synthetic retinoid EC23 (n = 16) or DMSO as the vehicle control (0.01%; n = 16) and all groups were operantly conditioned. A yoked control group incubated in Ch55 (10^{-6} M; n = 16) was also performed, once again to confirm that pharmacological treatment alone had no significant effect on respiratory behaviour. A mixed factor ANOVA revealed a significant interaction effect ($F_{(10,178)} = 5.95$; $p < 0.001$). As expected, yoked control animals incubated in Ch55 showed no significant change in their behaviour, either across the training sessions or the memory test (Figure 12A). Furthermore, as expected, DMSO-treated conditioned animals showed learning after two training sessions ($p < 0.001$) but did not form LTM at 30 hours ($p < 0.001$ MT relative to S2; Figure 12B). Animals incubated in the retinoid agonist Ch55 (Figure 12C), demonstrated learning, but also LTM at 30 hours following only two training sessions (ITM protocol);

the number of openings in the MT was not significantly different from S2, but was significantly different from the number of openings in the MT of both DMSO-conditioned ($p < 0.001$) and yoked controls ($p < 0.001$). Likewise animals incubated in PA024 (Figure 12D), AM80 (Figure 12E), and EC23 (Figure 12F) all demonstrated successful learning from S1 to S2 and demonstrated successful LTM formation at 30 hours, as the number of openings in the MT were not significantly changed from S2, and all were significantly different from the MT of the vehicle control. These data support our hypothesis that activation of retinoid receptors can promote memory formation in *Lymnaea*.

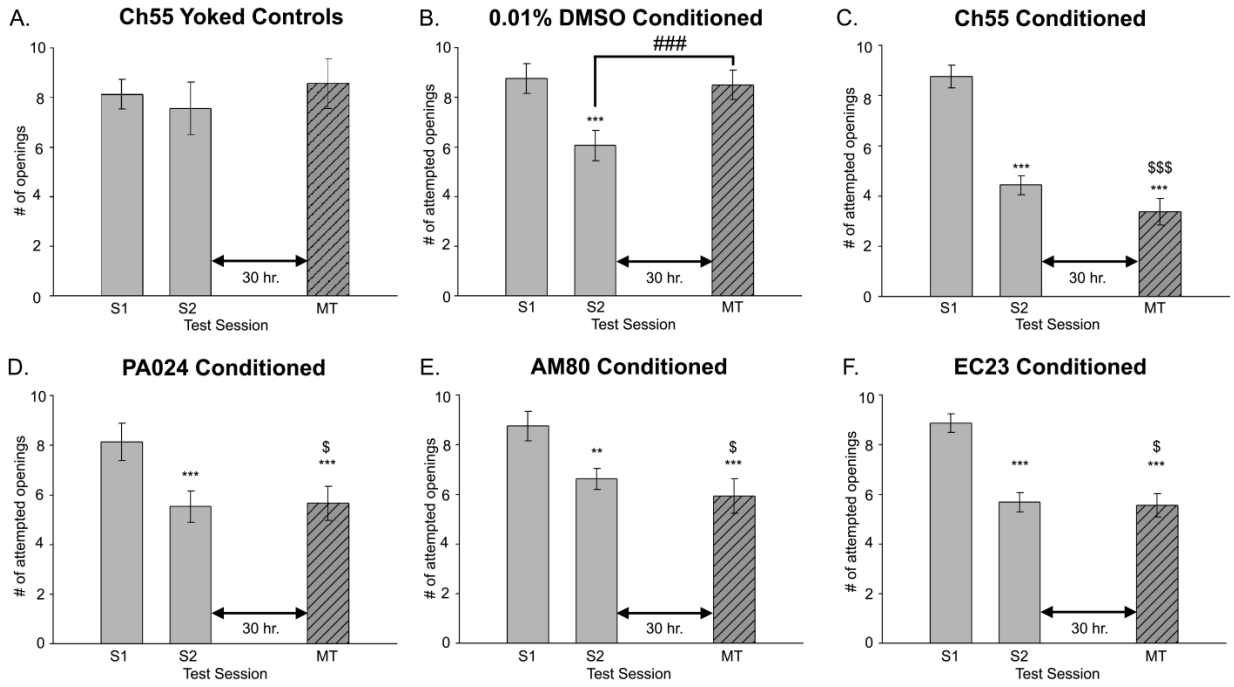

Figure 12. RXR and RAR agonists promote LTM formation. Animals were subjected to either the operant conditioning ITM procedure (two 45 minute training sessions) or to the yoked control procedure. The MT was administered 30 hours after training. All animals were incubated in either the vehicle (0.01% DMSO) or the retinoid receptor agonists (10^{-6} M) for a period of 24 hours before training (or yoked procedure) and until the MT. **(A)** Yoked control animals incubated in Ch55 (n = 16) showed no significant change in their behaviour across the training sessions or MT. **(B)** Animals incubated in 0.01 % DMSO (n = 16) and operantly conditioned, demonstrated learning but did not form LTM lasting 30 hours (*** $p < 0.001$ relative to S1; ### $p < 0.001$). **C-F.** Animals incubated in either **(C)** Ch55 (n = 16) **(D)** PA024 (n = 15), **(E)** AM80 (n= 16) or **(F)** EC23 (n = 16) all demonstrated both learning and LTM formation, 30 hours following operant conditioning. *** $p < 0.001$, ** $p < 0.01$ relative to S1. $ represents a significant difference compared to the MT of DMSO-treated conditioned animals ($$$ $p < 0.001$; $ $p < 0.05$).

2.05 Discussion

The importance of retinoic acid in vertebrate nervous system development and regeneration has long been recognized (Maden, 2007), but more recent studies have shed light on its role in the normal adult brain, where it is thought to be involved in hippocampal-dependent memories (Mey and McCaffery, 2004; Lane and Bailey, 2005). In this study, we now provide evidence that retinoid signaling also plays a role in implicit memory formation of an invertebrate species following operant conditioning.

Retinoic acid is important for vertebrate learning and memory

Behavioural studies in rodents have shown that both retinoid receptor mutants (Chiang et al., 1998; Wietrzych et al., 2005; Nomoto et al., 2012) as well as those maintained on vitamin A deficient diets (Cocco et al., 2002; Bonnet et al., 2008) have impaired spatial learning and memory (which is often reversed on re-instatement of vitamin A or retinoic acid). Vitamin A deficiency and/or disrupted retinoid signaling has also been shown to impair long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus (Chiang et al., 1998; Misner et al., 2001; Nomoto et al., 2012). Studies in adult songbirds have shown that RALDH activity in juvenile songbirds is required for song maturation (Denisenko-Nehrbass et al., 2000) and that application of retinoic acid to juvenile finches during the critical period for song learning, results in more variable songs in adulthood (Wood et al., 2008). These studies all suggest that retinoic acid activity in the CNS is important in vertebrate learning and memory.

Molluscs such as *Lymnaea* and *Aplysia* (Byrne et al., 1991; Benjamin et al., 2000; Parvez et al., 2006) have long been utilized to study cellular and molecular mechanisms underlying non-associative and associative learning, but to date, there has been no evidence supporting a role for

retinoid signaling in implicit memory formation in invertebrates. Although there is evidence that the ecdysone receptor of *Drosophila*, which heterodimerizes with the *Drosophila* homologue of RXR (*ultraspiracle*) plays a role in LTM formation in response to an edcysone metabolite (Ishimoto et al., 2009), experimental evidence suggests that *ultraspiracle* does not bind retinoic acid (Mangelsdorf et al., 1992). Thus, there is no evidence for retinoid signaling playing a direct role in LTM formation in *Drosophila*. In this study, we have now shown, using operant conditioning of the aerial respiratory behaviour of the mollusc *Lymnaea,* that retinoid signaling is important for successful LTM formation in an invertebrate species, and can even convert ITM into LTM.

Retinoid signaling in invertebrates

The genetic machinery for retinoid signaling is present not only in non-chordate deuterostomes (Cañestro et al., 2006), but also in protostomes (Simões-Costa et al., 2008; Albalat and Cañestro, 2009), including *Lymnaea*, suggesting an ancient origin of both retinoid metabolic enzymes (RALDH, Cyp26) and retinoid receptors (RAR and RXR). The CNS of *Lymnaea* contains retinoic acid (Dmetrichuk et al., 2008) which plays a role in both *Lymnaea* embryonic development (Carter et al., 2010; Carter, 2011), and neuronal outgrowth and survival (Dmetrichuk et al., 2006, 2008), similar to its role in vertebrates (Corcoran et al., 2000, 2002; Clagett-Dame et al., 2006). Other studies have also demonstrated a conserved role of retinoic acid in limb regeneration in invertebrates (Hopkins and Durica, 1995; Hopkins, 2001). Although the *Drosophila* homologue of RXR does not appear to bind retinoic acid, there is however, ample evidence for retinoid binding to other invertebrate RXRs. Specifically, the molluscan RXR of *Biomphalaria glabrata* (overall amino acid identity of 97% with *Lymnaea* RXR) can

transactivate transcription when treated with retinoic acid (Bouton et al., 2005) and the locust RXR also binds retinoic acid (Nowickyj et al., 2008).

In this study, we used two different pharmacological inhibitors of RALDH to interfere with retinoid signaling. Citral is a slow, non-competitive inhibitor of RALDH, whereas DEAB is a slow, competitive substrate (Koppaka et al., 2012). Their effects on organs are often rescued by application of exogenous retinoic acid (Marsh-Armstrong et al., 1994; Tanaka et al., 1996; Vandersea et al., 1998; Mathew et al., 2009). Yang et al. (2009) showed that citral induces reductions in retinoic acid levels in the hippocampus, as well as reducing spatial memory acquisition, further supporting retinoic acid's role in memory formation. In this study, we found that both RALDH inhibitors, as well as a number of retinoid receptor antagonists, inhibited LTM formation in *Lymnaea*. It is unlikely that any of the antagonists or inhibitors produced non-specific toxic effects on the animals because their initial respiratory behaviour (number of openings in session 1) was no different to that of control animals. Furthermore, toxicity would likely have resulted in reduced respiratory behaviour in the memory test, rather than the increase in behaviour seen here. Yoked control animals incubated in both HX531 and Ch55 also showed no significant changes in their respiratory behaviour across sessions. Interestingly, LE135 had absolutely no effect on memory, therefore acting as a positive control. LE135 is an antagonist selective for the RARβ sub-type, whereas the pan-antagonist LE540 is less selective than LE135 (Kagechika, 2002) but is apparently more potent (Umemiya et al., 1997; Kagechika, 2002). It is likely that *Lymnaea* has only one RAR, which shows ~ 55 % overall amino acid identity with all three vertebrate RAR sub-types (α, β and γ). This may explain why LE540 partially inhibited memory formation whereas LE135 had no effect. Both LE540 (RAR pan-antagonist) and PA452 (RXR pan-antagonist) appeared to be less effective at blocking LTM than HX531 (consistent with previous retinoid experiments in *Lymnaea*), but when combined

together, appeared to be more effective. This may suggest that both *Lym*RAR and *Lym*RXR are involved in LTM formation in *Lymnaea.* The potential role for both RAR and RXR receptors is also supported by vertebrate studies, where it has been shown that RARβ and RXRγ mutant mice show deficits in spatial learning tasks and hippocampal LTP (Chiang et al., 1998; Wietrzych et al., 2005). Our finding that the vertebrate receptor agonists selective for both RAR (AM80, Ch55, EC23) and RXR (PA024) all showed memory promoting capabilities, further supports this notion. We have previously shown that the RXR agonist, PA024 mimics the effects of retinoic acid on *Lymnaea* neurons (Carter et al., 2010), and that these effects are selectively inhibited by RXR pan-antagonists (HX531 and PA452), but not by the RAR pan-antagonist, LE540 (Rand, 2012). Though indirect evidence, these findings suggest that the receptor selectivity of these pharmacological agents is preserved in this species.

How does retinoic acid affect long-term memory formation?

The consolidation period between training sessions is thought to be necessary for LTM formation in *Lymnaea* (Lukowiak et al., 2000). We propose that it is during the actual training periods and the intervening consolidation periods, that retinoid signaling is required; incubation of animals in citral prior to (Figure 7), or during (Figure 8) these periods adversely affected LTM formation, whereas incubation of animals in citral after the completion of training (Figure 8) had no effect on LTM formation. Although LTM formation was inhibited, our data showed no effects of the RALDH inhibitors or receptor antagonists on ITM formation. Interestingly, Nomoto et al. (2012) also recently showed that long-term potentiation was disrupted, but short-term potentiation was normal in the hippocampus of transgenic mice expressing a dominant-negative RAR. Since ITM in *Lymnaea* is known to require RNA translation but not transcription, whereas LTM requires both (Sangha et al., 2003a), our data suggest that retinoic acid, binding to its

receptors, might be acting as a transcriptional activator during LTM consolidation in *Lymnaea.* We have previously shown non-nuclear distributions of the RXR and RAR in adult *Lymnaea* neurons (Carter et al., 2010; Carter, 2011), but it is possible that receptors present in the nucleus were below the detection limits of our Western blotting or immunostaining procedures. It is also possible that the retinoid receptors might translocate to the nucleus under certain conditions, or following activation (as shown previously during nerve injury, Schrage et al., 2006). An alternative explanation is that the retinoid receptors are not acting directly as transcription factors but are, instead, acting on cellular signaling cascades that initiate transcriptional processes downstream. Whether or not the retinoid receptors act as direct or indirect transcriptional activators in implicit memory formation in *Lymnaea* remains to be determined. Because the individual neurons mediating the respiratory behaviour in *Lymnaea* are identified (Syed et al., 1990) and are amenable to electrophysiological recordings as well as genetic analysis, and because many neuronal correlates of operant conditioning of the aerial respiratory behaviour have already been identified (reviewed in Spencer and Rothwell, 2013), this invertebrate model now holds future promise for investigations of how retinoic acid affects memory formation at a cellular and network level. The identified neuron, RPeD1, has previously been shown to be important for the transcriptional events taking place during LTM formation (Scheibenstock et al., 2002) and thus may be a possible target of retinoid signaling.

Which genes may be downstream targets of retinoic acid during memory formation in *Lymnaea* is also currently unknown. In juvenile songbirds, Wood et al. (2008), identified four genes whose expression changed as a result of retinoic acid supplementation (*brinp1, nrgn, RXRα, sdr2/scdr9*). Interestingly, *nrgn* encodes a postsynaptic calmodulin-binding protein required for calcium-dependent synaptic plasticity. Bonnet et al. (2008) have previously shown a decline in hippocampal TrkA expression following vitamin A deficiency (which was then

reversed following application of retinoic acid). Furthermore, expression of TrkB receptor was 3-fold higher following treatment of rat spinal cords with the RAR agonist, AM80 (Takenaga et al., 2009). With increasing evidence for the role of neurotrophic factors in learning and memory (Yamada et al., 2002; Shimazu et al., 2006; Bekinschtein et al., 2008; Bekinschtein et al., 2014), it is quite possible that genes encoding various neurotrophin receptors (also thought to contain retinoic acid response elements (Qi et al., 2013)), may be downstream targets of retinoid signaling during memory formation.

In summary, previous findings have indicated a role for retinoic acid in songbird learning and memory, as well as hippocampal-dependent memories in mammals. Our data now provide evidence for the role of retinoic acid in operant conditioning of an invertebrate species, and these findings suggest an ancient and conserved role for this molecule in memory formation.

2.06 Acknowledgements

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Chapter 3:

Novel interactive effects of darkness and retinoid signaling in the ability to form long-term memory following aversive operant conditioning

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J. Simmons ran the qPCR experiments shown in Figure 6 of the manuscript only (Figure 18 in this thesis).

G. Peters ran the experiment described on page 259 in the manuscript only (page 92 in this thesis).

3.01 Abstract

The vitamin A metabolite, retinoic acid, is important for memory formation and hippocampal synaptic plasticity in vertebrate species. In our studies in the mollusc *Lymnaea stagnalis,* we have shown that retinoic acid plays a role in memory formation following operant conditioning of the aerial respiratory behaviour. Inhibition of either retinaldehyde dehydrogenase (RALDH) or the retinoid receptors prevents long-term memory (LTM) formation, whereas synthetic retinoid receptor agonists promote memory formation by converting intermediate-term memory (ITM) into LTM. In this study, animals were exposed to constant darkness in order to test whether light-sensitive retinoic acid would promote memory formation. However, we found that exposure to constant darkness alone (in the absence of retinoic acid) enhanced memory formation. To determine whether the memory-promoting effects of darkness could override the memory-inhibiting effects of the retinoid signaling inhibitors, we exposed snails to RALDH inhibitors or a retinoid receptor antagonist in constant darkness. We found that darkness overcame the inhibitory effects of RALDH inhibition, but did not overcome the inhibitory effects of the retinoid receptor antagonist. We also tested whether constant darkness and training affected the mRNA levels of the retinoid metabolic enzymes RALDH and Cyp26, or the mRNA levels of the retinoid receptors, but found no significant effect. Overall, these data demonstrate an interaction between environmental light conditions and the retinoid signaling pathway, which influence long-term memory formation in a mollusc.

3.02 Introduction

The aerial respiratory behaviour of the mollusc, *Lymnaea stagnalis*, can be operantly conditioned to produce intermediate-term memory (ITM) or long-term memory (LTM), depending on the number of training sessions used (Lukowiak et al., 1998, 2000). Over the last 20 years, numerous studies have determined how various factors such as age, stress and exposure to toxins can affect the ability of these animals to form long-term memories (reviewed by Spencer and Rothwell, 2013; Lukowiak et al., 2014). More recently, we have discovered that the vitamin A metabolite, retinoic acid, is important for the formation of long-term memory following operant conditioning of the respiratory behaviour of *Lymnaea* (Rothwell and Spencer, 2014). Studies in vertebrates have also shown that vitamin A and retinoic acid are important in memory formation (Chiang et al., 1998; Cocco et al., 2002; Wietrzych et al., 2005; Bonnet et al., 2008; Nomoto et al., 2012), and disruption of retinoid signaling pathways can have detrimental effects on hippocampal long-term potentiation (LTP) and depression (LTD) (Chiang et al., 1998; Misner et al., 2001; Nomoto et al., 2012).

In both the canonical and reported "ancestral" pathways of retinoic acid synthesis, the final irreversible oxidation of retinal to retinoic acid is carried out by the enzyme retinaldehyde dehydrogenase (RALDH). Retinoic acid enters the nucleus where it binds to retinoid receptors which are members of the nuclear hormone receptor family. There are two classes of retinoid receptors, the retinoid X receptors (RXRs) and retinoic acid receptors (RARs), which bind to retinoic acid response elements (RAREs) in the promoter region of target genes and act as ligand-activated transcription factors to regulate gene expression. Endogenous retinoic acid is then broken down by the enzyme Cyp26. We have previously shown that the CNS (and hemolymph) of *Lymnaea* contains physiologically relevant concentrations of retinoic acid and have previously demonstrated RALDH activity in CNS extracts (Dmetrichuk et al., 2008). We

have also cloned RALDH (Genbank Accession no. FJ539101), Cyp26 (Genbank Accession no. KF669878), an RXR (Carter et al., 2010; Genbank Accession no. AY846875) and a putative RAR (Carter, 2011; Genbank Accession no. GU932671) from the *Lymnaea* CNS. Though retinoid signaling has apparently been lost in many protostome lineages, it appears to be intact in many molluscan species (Bouton et al., 2005; Simões-Costa et al., 2008; Albalat and Cañestro, 2009), including *Lymnaea*.

Previous experiments have shown that pharmacological inhibitors of RALDH and retinoid receptors can prevent LTM formation in *Lymnaea*, while having no effect on either learning or ITM. Furthermore, treatment with retinoid receptor agonists promoted the formation of LTM (Rothwell and Spencer, 2014). In the previous study, only synthetic retinoid agonists were used, so the initial aim of this study was to determine whether exogenous application of the natural ligand, retinoic acid, could also promote memory formation following operant conditioning of the aerial respiratory behaviour. However, a serendipitous discovery that incubation of animals in constant darkness (because of the light-sensitive nature of retinoic acid) enhanced memory formation in these animals, led us to investigate how this memory-enhancing effect of darkness might interact with retinoid signaling. Our main aim was thus to combine two opposing treatments: one memory-enhancing (darkness), the other memory-inhibiting (inhibition of retinoid signaling), to determine which one would prevail.

It is well known that circadian rhythms affect memory formation in a number of different diurnal species, including the mollusc *Aplysia* (Lyons et al., 2005; Lyons et al., 2006; Lyons, 2011), with animals learning better during the day than at night. *Lymnaea* are more active in the daytime and have also demonstrated more effective acquisition of a conditioned behaviour in the morning than in the afternoon (Wagatsuma et al., 2004). Many of these studies have used constant darkness in their protocols, yet to our knowledge, there has been no previous evidence

that such conditions might promote LTM formation. Interestingly, it has also been shown that photoperiod can affect retinoid metabolism and affect levels of both RALDH and Cyp26 (Helfer et al., 2012), though no studies have previously reported the effects of constant darkness on retinoid enzyme levels. In this study, we first demonstrated that the memory-enhancing effect of constant darkness was able to override any inhibitory effects of RALDH inhibition on LTM formation, but was not able to override the inhibitory effects of a retinoid receptor antagonist. Using real-time quantitative PCR, we then determined whether constant darkness (with and without training), affected the mRNA levels of either RALDH or Cyp26, as well as the retinoid receptors, in the *Lymnaea* CNS.

3.03 Materials and Methods

Animals

Lymnaea stagnalis (originally obtained from stocks at the Vrije University, Amsterdam) were bred in the laboratory environment and maintained in dechlorinated water at room temperature on a 12:12 hour fixed light-dark cycle. *Lymnaea* were fed a combination of romaine lettuce leaves and NutraFin Max Spirulina fish food (Hagen). Adult animals ranging in shell length from 22 to 30 mm were used for all experimental procedures. Prior to the initiation of any experimental procedures, *Lymnaea* were labeled with coloured markings on their shells and were permitted to freely perform aerial respiration.

Chemicals

All-*trans* retinoic acid (atRA) was obtained from Sigma-Aldrich and prepared in 100 % EtOH, and then diluted in water to a final concentration of 10^{-6} M (0.01 % EtOH). Vehicle control experiments were performed with 0.01% EtOH. The RALDH inhibitors, citral and 4-diethylaminobenzaldehyde (DEAB) were obtained from Sigma-Aldrich and stock solutions were prepared in 70 % EtOH. Citral was diluted to a final concentration of 50 µM (0.35 % EtOH) and DEAB was diluted to a final concentration of 100 µM (0.35 % EtOH) in water. Vehicle control experiments were performed with 0.35 % EtOH. The RXR pan-antagonist, HX531, was a generous gift from Dr. H. Kagechika (University of Tokyo, Japan) and was prepared in 100 % DMSO. HX531 was diluted to a final concentration of 10^{-6} M in water. Vehicle control experiments were run in 0.01 % DMSO.

Operant conditioning of aerial respiration

The aerial respiratory behaviour of *Lymnaea* was operantly conditioned as previously described (Lukowiak et al., 1996, 2000; Khan and Spencer, 2009; Rothwell and Spencer, 2014). Animals were trained in a 1 L test beaker containing 800 mL of hypoxic water (water bubbled with 100 % N_2 gas for 20 minutes prior to and during all training sessions and memory tests) in order to encourage the performance of aerial respiration over cutaneous respiration (Lukowiak et al., 1996). *Lymnaea* were given 30 minutes to acclimate to the hypoxic environment before session 1 (S1) (unless otherwise stated) and 10 minutes before all other training sessions and the memory test. Snails were gently propelled to the bottom of the test beaker to mark the initiation of each training session and memory test. All training sessions were 45 minutes in duration with a 1 hour consolidation period between sessions. Operantly conditioned animals received a tactile stimulus to the open pneumostome each time aerial respiration was attempted during each training session. This stimulus was sufficient to induce immediate pneumostome closure without inducing the whole body withdrawal response. Yoked control animals received the same number of tactile stimuli as the operantly conditioned animals, but the tactile stimulus was applied to the closed pneumostome each time the animal to which it was yoked, opened its pneumostome. Thus in the yoked control group, stimulus application was not contingent upon pneumostome opening. The number of attempted pneumostome openings was recorded for each operantly conditioned animal, while the number of pneumostome openings was recorded for the yoked control groups. Snails were placed in eumoxic home tanks or incubation beakers between training or testing sessions.

To produce intermediate-term memory (ITM) lasting 2 hours, two 45 minute training sessions (S1 and S2) were administered, with a 1 hour consolidation period between sessions. The memory test (MT) was administered 2 hours after S2. A separate group of animals were also tested 24 hours after S2 to test for long-term memory (LTM).

To produce LTM lasting 24 hours, four 45 minute training sessions (S1 to S4) were administered with 1 hour consolidation periods between training sessions. A MT was conducted 24 hours after the final training session and the conditioned animals were again stimulated on the open pneumostome during the MT. LTM was also assessed using 30 minute freely-breathing pre-test and post-test sessions before and after the training procedure respectively. During these freely-breathing sessions, the number of pneumostome openings was recorded for each snail and the number of pneumostome openings observed during the post-test was compared to the behaviour observed during the pre-test.

Procedure for exposure to darkness

Animals were randomly assigned to either a 'darkness' group or to a control group. The darkness group was maintained in constant darkness prior to S1, between all training sessions, and until the MT, whereas the animals in the control group were maintained on the normal 12:12 hour light-dark cycle. To assess the influence of darkness on memory formation in *Lymnaea* two different approaches were used. In the first approach, the animals were placed in a eumoxic home tank under a plastic box (18 in. x 12 in. x 24 in.). For the 'darkness' group, this was an opaque black box which shielded the snails from any light in the surrounding environment. For the control group, animals were placed in a box identical in size and thickness to the dark box. However, this box was made of clear, translucent plastic and therefore allowed animals to be maintained on the normal 12:12 hour light-dark cycle (but controlled for any build-up of gases or temperature).

In the second approach, snails were maintained in an open lab environment. The 'darkness' group was maintained under constant darkness in an unused, window-less laboratory,

while the controls were maintained on the normal 12:12 hour light-dark cycle within a separate, but similar laboratory setting (similar temperature and size).

Animals were maintained in constant darkness for 48 hours (unless stated otherwise) prior to the initiation of operant conditioning (for either the enclosed box or open-lab protocols). All training sessions and memory tests were conducted in the light, and animals were returned to the dark environment between all training sessions, until the MT.

Procedures for chemical incubations

I) All-trans RA:

Retinoic acid is photosensitive, so incubations were conducted under constant darkness to prevent its degradation. Snails were randomly assigned to one of three groups and incubated in either i) 500 mL of atRA (10^{-6} M), ii) 500 mL of 0.01 % EtOH, or iii) 500 mL of pond water and maintained in constant darkness (in an enclosed environment). Incubations started 24 hours before the first training session (S1) and were maintained until the MT. Animals were removed from the incubation beakers and placed in the hypoxic test beaker during all training sessions and the MT. All animals were operantly conditioned using two 45 minute training sessions and a MT was administered 24 hours after S2 to assess for LTM. The number of attempted pneumostome openings was recorded during each training session and the MT.

II) RALDH inhibitors:

Animals were randomly assigned to different groups and incubated in either i) 500 mL of the RALDH inhibitor citral (50 μM), ii) 500 mL of the RALDH inhibitor DEAB (100 μM), or iii) 500 mL of 0.35 % EtOH, as a vehicle control. For each of these conditions, snails were either maintained in constant darkness, or on a normal 12:12 hour light-dark cycle prior to being

operantly conditioned. We have previously shown that incubation in citral for 48 hours (Rothwell, 2008) or 72 hours (Rothwell and Spencer, 2014) prior to S1, results in impaired LTM formation. For this study, all incubations were initiated 48 hours before the first training session (S1). Animals were placed in the hypoxic test beaker at the initiation of S1, but were returned to their respective incubation beakers between training sessions and until the MT. *Lymnaea* were operantly conditioned with four training sessions, and the number of attempted pneumostome openings was recorded during each training session. The MT was conducted 24 hours later to test for LTM.

III) RXR pan-antagonist HX531:

Animals were incubated in either 200 mL of the RXR pan-antagonist HX531 (10^{-6} M) or 200 mL of 0.01 % DMSO (vehicle control) in aerated 400 mL beakers for 48 hours before the first training session (S1). For each incubation condition, the animals were exposed to either constant darkness, or the normal 12:12 hour light-dark cycle for 48 hours prior to S1. Training was performed as stated above for the RALDH inhibitors (four training sessions and a MT 24 hours later).

Righting response and exploratory behaviour testing

Lymnaea were maintained in dechlorinated water for 48 hours (unless stated otherwise) in either constant darkness or the normal 12:12 hour light-dark cycle in an open environment. Prior to the start of the behavioural tests, all animals were given a 20 to 30 minute acclimation time in the light (consistent with that given prior to operant conditioning). All tests were carried out in 8.5 cm Petri dishes containing clean pond water and each animal was tested in a separate dish. Each animal was given only one test (either the righting response or the exploratory

behaviour test) and the order in which the animals were tested was alternated between dark and light-exposed animals. Procedures were carried out in a similar manner to those in Orr et al. (2007).

At the beginning of each righting response test, an animal was placed in a Petri dish and inverted onto the dorsal side of its shell, exposing its foot. The time taken for the animal to right itself was recorded. Each animal was tested three times, with 20 minutes in between each test. An average response time was obtained for each animal and a t-test was used to test for significant differences between dark and light-exposed animals.

At the beginning of each exploratory behaviour test, an animal was placed in a Petri dish at which time it performed the full-body withdrawal response. The time taken for the animal to emerge from its shell and begin locomotion was recorded. Each animal was tested three times, with 20 minutes in between each test. An average response time was obtained for each animal and a Mann-Whitney Rank Sum test was used to test for significant differences.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from adult *Lymnaea* CNS using Animal Tissue RNA purification kit from Norgen Biotek (Thorold, ON, Canada). RNA extracted from a pooled sample of four CNS represented each biological replicate. The iScript™ cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada) was used to synthesize cDNA from 1 µg of total RNA. A no reverse transcriptase control was used to confirm the absence of genomic DNA contamination in RNA samples. qPCR reactions were performed using the iQ™ SYBR® Green Supermix (Bio-Rad) and a CFX96™ thermal cycler (Bio-Rad) and all protocols were carried out following MIQE guidelines (Bustin et al., 2009). Three technical replicates of three biological replicates were performed for each condition. Gene-specific qPCR primer pairs were designed for four

normalizer genes, Eukaryotic Initiation Factor-4α (EIF4α), β-tubulin, actin and 18srRNA, and the four genes of interest, *Lym*RALDH, *Lym*Cyp26, *Lym*RXR and *Lym*RAR. The primers used for RALDH, Cyp26, RXR and RAR were as follows: RALDH (forward primer: TAGCGCAAGAGGTCGAT; reverse primer: CATCCTGCATAGTACCTGAAC); Cyp26 (forward primer: TGGTGCTGATTACATTCGG; reverse primer: CAGGTAGTCGTCCAGTCT); RXR (forward primer: GTTCGTCGAGAGGTAAACCAG; reverse primer: CCATGGACATTCCACCAGACA); RAR (forward primer: CATCCGCTATGAGCTTGACA; reverse primer: GCATGGCGTACTCTGTCTCA). Standard curves were used to demonstrate that the amplification efficiency of each primer pair was 100 ± 5%. No template controls were run for each primer pair. The CFX Manager software (Bio-Rad) was used to calculate relative normalized expression using the ΔΔCq method (Vandesompele et al., 2002; Hellemans et al., 2007).

Data and statistical analysis

In this study learning was operationally defined as a significant reduction in the number of attempted pneumostome openings from the first training session to the final training session (Lukowiak et al., 1996; Rothwell and Spencer, 2014) and a significant reduction in the number of openings in the final training session compared to that of yoked controls. Memory formation was deemed to have occurred when a) the number of attempted pneumostome openings in the MT was not significantly different from that observed in the final training session and b) the number of attempted pneumostome openings in the MT remained significantly lower than that observed in the first training session (Lukowiak et al., 1998; Rothwell and Spencer, 2014), as well as significantly lower than the MT of yoked controls. For the freely-behaving pre-test and post-test sessions, memory was defined as a significant reduction in pneumostome opening in the post-test session relative to the pre-test session.

A mixed factor ANOVA was used to analyze all behavioural data following operant conditioning (unless stated otherwise), where condition was specified as the fixed factor and session as the repeated, random factor. A Tukey *post-hoc* test was used for comparisons, which were deemed significant when $p < 0.05$. All data are presented as mean ± SEM. An unpaired t-test was used to test for significant differences in relative mRNA levels between darkness and light conditions.

3.04 Results

Incubation in retinoic acid, as well as darkness, promotes LTM formation

We have previously shown that inhibitors of RALDH, as well as retinoid receptor antagonists, inhibit the formation of LTM following operant conditioning of the aerial respiratory behaviour. Furthermore, we showed that retinoid receptor agonists were able to promote the formation of LTM, using a training protocol that normally produces only ITM lasting 2 hours (Rothwell and Spencer, 2014). Our first aim was to determine whether animals incubated in exogenously applied retinoic acid (atRA) would also show LTM at 24 hours, following use of the ITM training protocol.

We first demonstrated that the ITM protocol, consisting of two 45 minute training sessions, did indeed only produce ITM at 2 hours, but not LTM at 24 hours, as shown previously (Rothwell and Spencer, 2014). Following two 45 minute training sessions (S1 and S2) with 1 hour in between, a MT was administered either 2 hours later (to assess for ITM) or, for a separate group of animals, 24 hours later (to assess for LTM) (Figure 13Ai). The behaviour in the MT at 2 hours (normalized to that of S1) was significantly reduced in the operantly conditioned group ($n = 13$) compared to the yoked control group ($n = 13$) (Figure 13Aii; t-test, $p < 0.01$). As expected from previous studies, two training sessions did not however lead to LTM formation at 24 hours, as the behaviour in the operantly conditioned group ($n = 16$) during the 24 hour MT (normalized to that of S1), was not significantly different from that of yoked controls ($n = 16$) (Figure 13Aiii; t-test, $p > 0.05$). We thus confirmed that two 45 minute training sessions (ITM training protocol) produced memory lasting 2 hours (ITM), but not memory lasting 24 hours (LTM). Using this ITM training protocol, we next determined whether incubation in atRA could promote ITM into LTM.

Animals were incubated in either atRA (10^{-6} M) or 0.01 % EtOH (as a vehicle control) for 24 hours prior to being operantly conditioned. Since atRA is photosensitive, this incubation needed to be conducted in the dark which introduced an additional experimental variable. A separate group of animals was thus maintained in pond water in the dark as an additional control. All animals were trained with the ITM procedure (two training sessions) and the MT was administered 24 hours after S2 in order to test for LTM (Figure 13Bi). A mixed factor ANOVA revealed a significant difference across sessions ($F_{(2,90)} = 29.31$; $p < 0.001$). Animals incubated in atRA ($n = 16$) showed a significant reduction in pneumostome openings from S1 to S2 ($p < 0.05$) and thus demonstrated learning (Figure 13Bii). This reduction in behaviour also lasted for 24 hours (MT vs. S1: $p < 0.01$), suggesting that exposure to atRA produced LTM. Animals incubated in EtOH ($n = 17$, Figure 13Biii), as well as pond water ($n = 15$, Figure 13Biv) and maintained in constant darkness, also both demonstrated significant reductions in openings between S1 and S2 ($p < 0.01$), demonstrating learning. Surprisingly, we discovered that both control groups maintained in constant darkness in EtOH and pond water also maintained this reduction in behaviour for 24 hours, thus showing LTM (MT vs. S1: EtOH $p < 0.001$; pond water $p < 0.05$). Figure 13C illustrates significant differences in behaviour during the 24 hour memory test (normalized to S1) for conditioned groups in the darkness as well as conditioned and yoked control groups in the normal light-dark cycle ($p < 0.001$; Kruskal-Wallis 1-way ANOVA on ranks). Together, these data suggest that exposure to constant darkness prior to training promoted LTM formation.

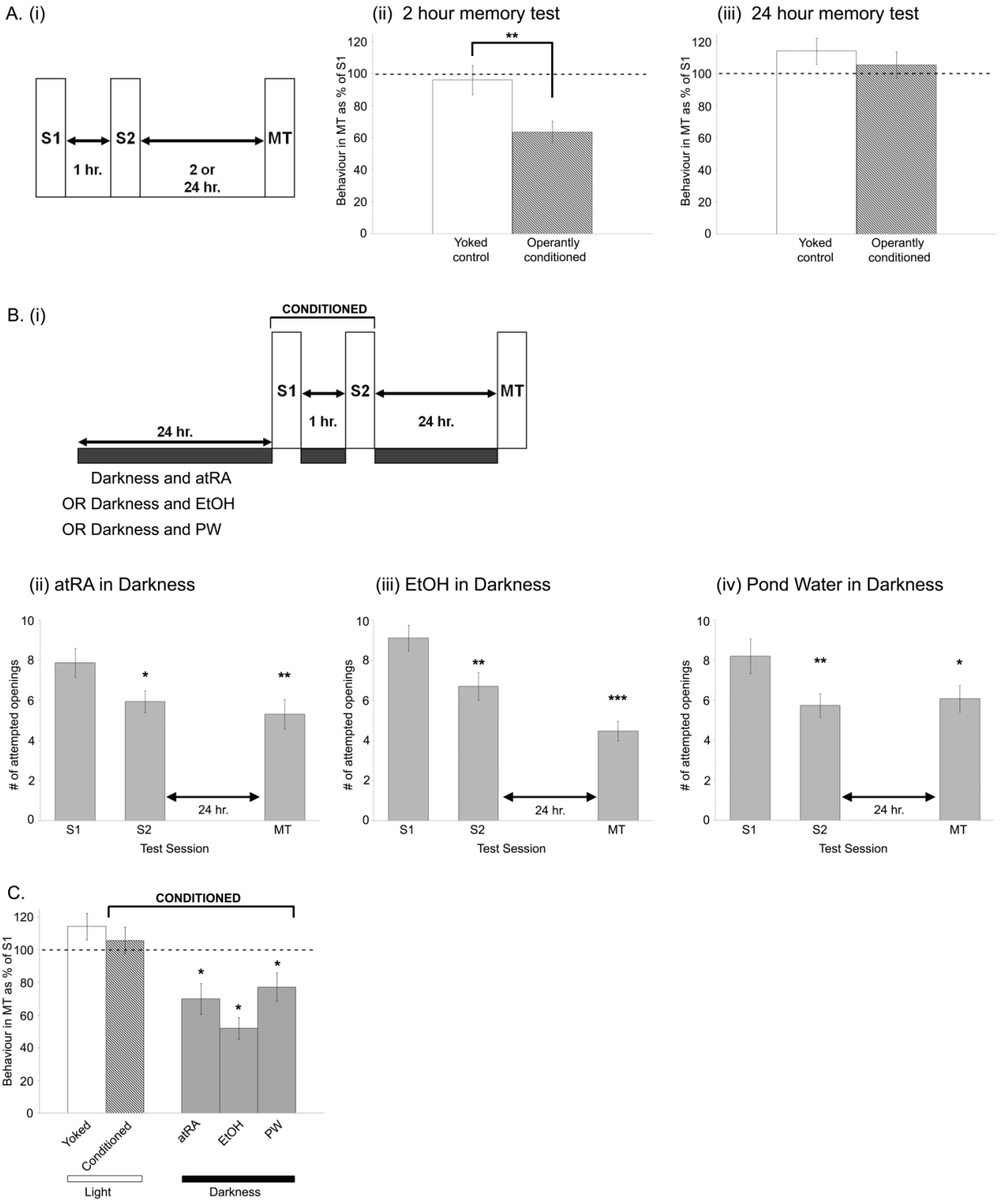
A. (i)
S1
S2
MT
1 hr.
2 or
24 hr.
(ii) 2 hour memory test
(iii) 24 hour memory test
Behaviour in MT as % of S1
Yoked control
Operantly conditioned
B. (i)
CONDITIONED
24 hr.
Darkness and atRA
OR Darkness and EtOH
OR Darkness and PW
(ii) atRA in Darkness
(iii) EtOH in Darkness
(iv) Pond Water in Darkness
# of attempted openings
Test Session
C.
Yoked
Conditioned
atRA
EtOH
PW
Light
Darkness

Figure 13.

Figure 13. The effects of retinoic acid on memory formation following two training sessions. **(Ai)** All animals received two training sessions spaced 1 hour apart. A memory test was administered either 2 hours or 24 hours after the last training session. All animals for this experiment were maintained on the 12:12 hour light-dark cycle. **(Aii)** When memory was assessed two hours after S2, behaviour in the MT (normalized to S1) was significantly reduced in the operantly conditioned group (n = 13) relative to the yoked controls (n = 13; ** $p < 0.01$). **(Aiii)** When memory was assessed 24 hours after S2, behaviour in the MT (normalized to S1) was not significantly different between operantly conditioned animals (n = 16) and yoked controls (n = 16; $p > 0.05$) **(Bi)** Animals were incubated in atRA, EtOH or pond water in constant darkness 24 hours before the initiation of training. All animals were conditioned with two training sessions and LTM was assessed 24 hours after S2. **(Bii)** Animals incubated in atRA (n = 16) in the dark for 24 hours, and then given two 45 minute training sessions demonstrated learning, but also LTM at 24 hours. Animals incubated in EtOH **(Biii**; n = 17**)** or pond water alone **(Biv**; n = 15**)** and exposed to darkness for 24 hours, also demonstrated LTM at 24 hours. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to S1). **(C)** Graph showing the behaviour in the 24 hour MT (normalized to S1) in animals from all experimental conditions illustrating the significant reduction in behaviour in all animals exposed to constant darkness, regardless of the pharmacological treatment. (* $p < 0.05$ relative to light-yoked controls; Kruskal-Wallis 1-way ANOVA on ranks with Dunn's *post-hoc* test performed in **C** only).

Exposure to darkness prior to training enhances memory formation

Our initial results above suggested that constant exposure to darkness was sufficient to enhance memory formation in *Lymnaea*. However, all experimental groups used above (Figure 13Bii-iv) were maintained under a black box and so we could not rule out that a build-up of gases or temperature may have played a role in the observed change in behaviour. Next, we designed experiments to specifically test the effects of darkness in promoting LTM using two different approaches. In the first, we designed two boxes under which *Lymnaea* were housed, which were identical in nature except one was black (for constant darkness), whereas the other was translucent (allowing for a normal light-dark cycle). In the second approach, we housed animals in an open lab environment, one group in constant darkness, the other group in normal 12:12 hour light-dark conditions.

In the first approach, animals were housed in pond water under one of the two boxes for 48 hours before training; one group under the dark box, the other group under the translucent box. Both groups were operantly conditioned using the ITM paradigm (two training sessions), but tested for LTM at 24 hours. A mixed factor ANOVA revealed a significant interaction effect between condition and session ($F_{(2,72)} = 7.33$; $p = 0.001$). The animals exposed to the normal light-dark cycle ($n = 19$) demonstrated learning, with a significant reduction in the number of attempted pneumostome openings in S2 compared to S1 ($p < 0.001$). These animals did not however form LTM lasting 24 hours, as the number of openings during the MT was significantly greater than seen during S2 ($p < 0.01$) but not significantly different from that seen during S1 ($p > 0.05$; Figure 14A). Animals maintained in constant darkness ($n = 19$) also demonstrated learning, with a significant reduction in pneumostome openings from S1 to S2 ($p < 0.001$). However, unlike the animals contained under the translucent box, the animals maintained in constant darkness formed LTM lasting 24 hours ($p < 0.001$ relative to S1) (Figure 14A).

We next repeated these experiments using open lab environment conditions; one group was maintained in constant darkness (n = 13) whereas the other was maintained on the normal light-dark cycle (n = 12). Both groups were operantly conditioned with the two session ITM paradigm and tested for LTM at 24 hours. A mixed factor ANOVA revealed a significant difference between conditions ($F_{(1,46)} = 11.23$; $p = 0.003$). Overall, the data obtained from the open environments showed the same results as those from the enclosed (box) environments above. That is, only the animals maintained under constant darkness for 48 hours prior to S1 demonstrated LTM after 24 hours (Figure 14B; MT vs. S1 $p < 0.01$). A yoked control group (n = 13) maintained in constant darkness was also given two sessions and a 24 hour MT, and showed no significant change in behaviour between S1 and the MT ($p > 0.05$) confirming that darkness alone had no significant effect on respiratory behaviour (Figure 14C). The behaviour during the MT (normalized to S1) of animals exposed to constant darkness, was significantly reduced (t-test, $p < 0.05$) in the operantly conditioned group, but not in the yoked controls (Figure 14C). These findings suggest that 48 hours of constant darkness prior to the initiation of operant conditioning, enhanced memory formation in *Lymnaea*. Because very similar results were obtained using both boxed and open-environment incubations, these data demonstrate that keeping animals in the boxed environments did not influence their aerial respiratory behaviour, thus validating the use of either experimental approach.

Longer exposures to darkness also promote LTM

It has previously been shown that exposure to stressors (e.g. predator effluent) promotes memory formation following operant conditioning of the respiratory behaviour in *Lymnaea*, resulting in LTM using only an ITM training protocol (Orr and Lukowiak, 2008; Orr et al., 2009; Dalesman et al., 2011a). These previous findings resemble the results seen above, so we

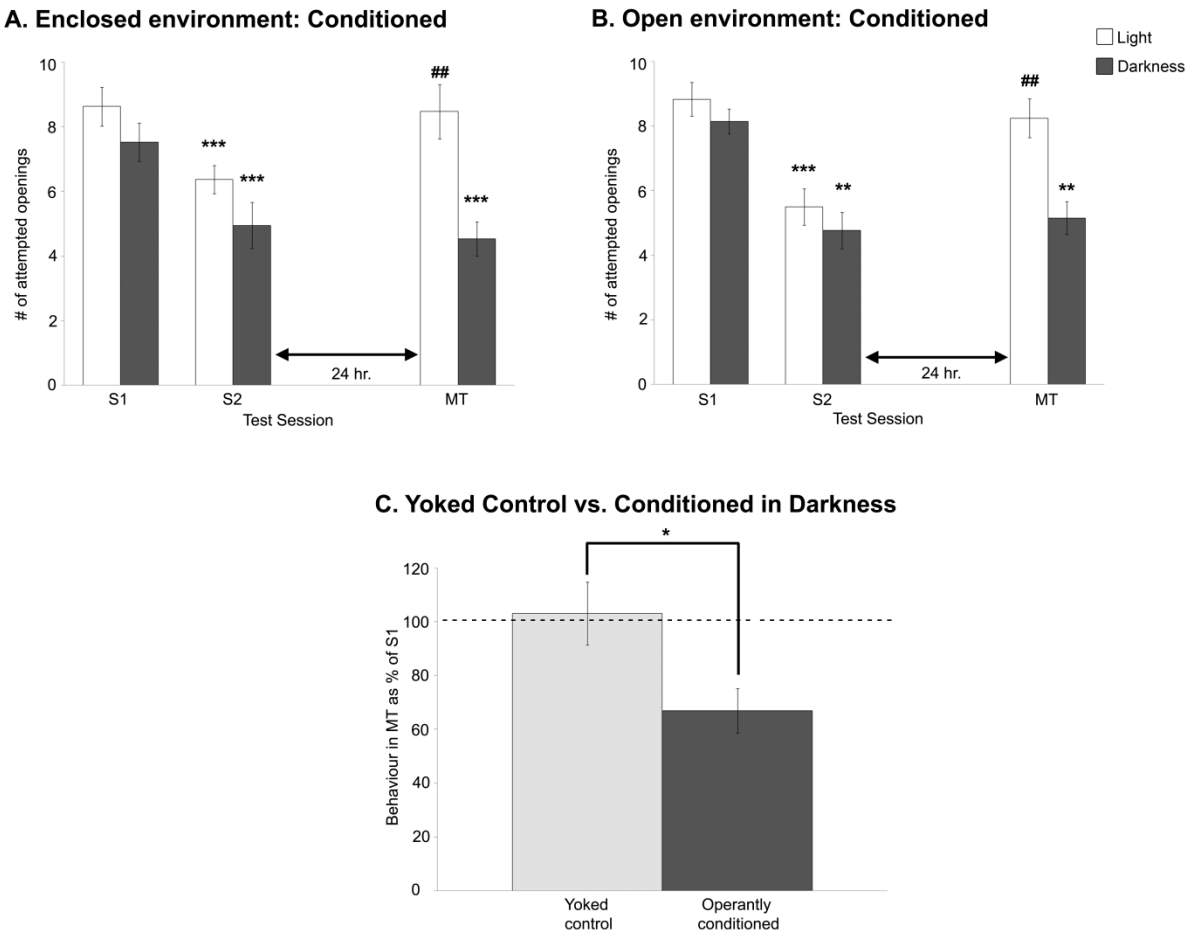

Figure 14. Exposure to constant darkness promotes LTM formation. Animals were exposed to 48 hours of constant darkness or a 12:12 hour light-dark cycle (light) using either boxes **(A**: enclosed environment**)** or an open environment **(B)** Animals were then conditioned using two 45 minute sessions and their behaviour in the 24 hour MT analyzed. All animals demonstrated learning, regardless of their incubation treatment. However, animals exposed to the normal light-dark cycle either in the enclosed (**A;** n = 19) or open (**B;** n = 12) environment, all failed to show LTM in the 24 hour MT. All animals exposed to constant darkness, either in the enclosed environment **(A;** n = 19**)** or in the open environment **(B;** n = 13**)** demonstrated a significant reduction in their behaviour in the 24 hour MT and thus exhibited LTM. (** $p < 0.01$, *** $p < 0.001$ relative to S1; ## $p < 0.01$ relative to S2). **(C)** Behaviour in the MT was normalized to that of S1. Following 48 hours of constant darkness in the open environment, operantly conditioned animals demonstrated a significant reduction in behaviour in the 24 hour MT compared to yoked controls. (* $p < 0.05$). These data clearly demonstrate that only animals exposed to constant darkness and conditioned with two training sessions showed a reduction in behaviour at 24 hours.

hypothesized that exposure to constant darkness may have acted as a stressor to these animals. We therefore tested two behaviours known to be significantly affected by other forms of stress to *Lymnaea* (such as predator detection; Orr et al., 2007). The first behaviour tested was the righting response in which the animal, when placed on the dorsal surface of its shell, will right itself to regain contact with the substrate. It was previously shown that animals exposed to the stress of predator detection reduce the time taken to perform this behaviour (Orr et al., 2007). We found however, that there was no significant difference ($p = 0.619$) in the righting response of animals exposed to 48 hours of constant darkness ($n = 19$) compared to those exposed to the normal light-dark cycle ($n = 19$; Figure 15Ai). We also tested the time taken for animals to begin exploratory behaviour following full-animal withdrawal. Previously, *Lymnaea* exposed to the stress of predator detection increased their time to exploration (Orr et al., 2007). Once again however, we found no significant difference ($p = 0.877$) between the time taken to start exploratory behaviour for animals exposed to 48 hours of constant darkness ($n = 17$) compared to those exposed to the normal light-dark cycle ($n = 17$; Figure 15Aii). These findings suggest that exposure to 48 hours of constant darkness was not acting as a stressor. However, it is possible that the righting and exploratory behaviours affected by the stress of predator detection are not affected to the same extent by other forms of stress.

If 48 hours of constant darkness was acting as a stressor, we hypothesized that animals might eventually habituate to this stress over much longer periods of darkness and as a result, would no longer exhibit memory enhancement. We next exposed animals to 7 days of constant darkness ($n = 10$) or normal light-dark cycles ($n = 10$). We then operantly conditioned the respiratory behaviour of all animals using the ITM procedure (2 training sessions) and tested for LTM formation 24 hours later (Figure 15Bi). A mixed factor ANOVA revealed a significant interaction between condition and session ($F_{(2,36)} = 6.97$; $p < 0.005$) and a *post-hoc* test revealed that the animals exposed to constant darkness for 7 days exhibited a significant reduction in their

pneumostome openings in the MT compared to S1 ($p < 0.01$), thus showing LTM formation (Figure 15Bii). As expected, animals exposed to the normal light-dark cycle did not exhibit LTM. These experiments demonstrate that exposure to constant darkness over 7 days was still able to promote LTM formation in *Lymnaea*. We also tested a separate group of animals incubated in darkness for 7 days for any changes in their righting response or exploratory behaviour, but once again found no significant differences compared to animals maintained on the normal light-dark cycle (data not shown). Overall these data support our earlier findings that a stress response in unlikely to be involved.

Exposure to darkness overcomes memory impairment induced by RALDH inhibitors

We have previously shown that the RALDH inhibitors, citral and DEAB, inhibit LTM formation that normally occurs after four 45 minute training sessions (Rothwell and Spencer, 2014). Our next aim was to use this same 4-session training procedure to determine how the memory-enhancing effects of darkness might interact with the memory-inhibiting effects of citral and DEAB. First however, we confirmed that four 45 minute training sessions would again produce LTM under normal (pond water) training conditions following a 12:12 hour light-dark cycle. Animals were subjected to either operant conditioning or the yoked control procedure and a mixed factor ANOVA revealed a significant interaction effect between condition and session ($F_{(2,44)} = 7.10$; $p = 0.002$). Only operantly conditioned animals ($n = 12$), but not yoked controls ($n = 12$), demonstrated a significant reduction in pneumostome openings from S1 to S4 to show learning ($p < 0.001$). This reduction in behaviour was maintained over 24 hours in the conditioned group only, to show LTM formation after 24 hours (Figure 16Ai; $p < 0.001$).

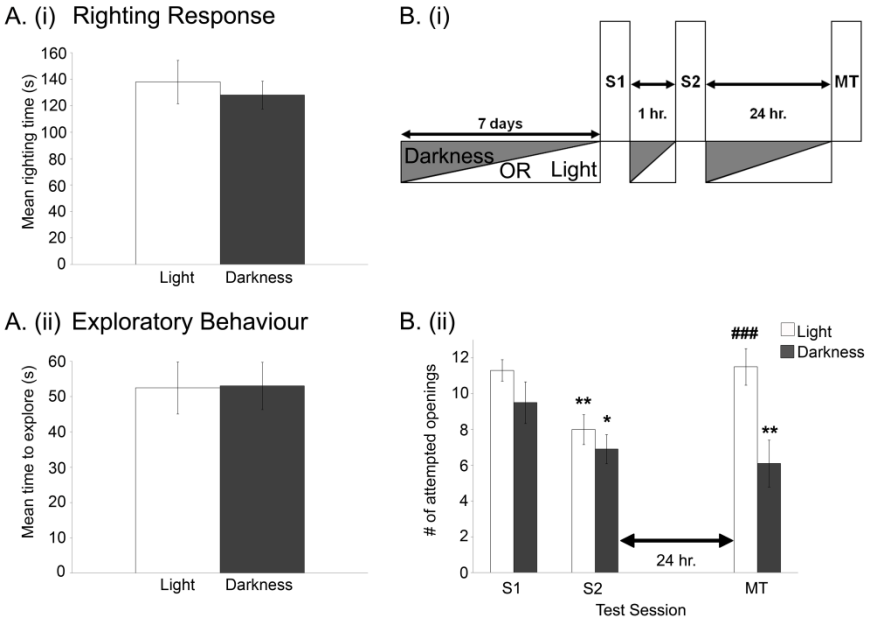

Figure 15. Constant darkness over 7 days promotes memory formation. (A) Following 48 hours of exposure to either darkness or the 12:12 hour normal light dark cycle, animals were given behavioural tests that examined either the righting response (**i**; n = 19) or the exploratory behaviour (**ii**; n = 17). No significant differences were found between the two conditions for either behaviour (righting response: t-test, $p > 0.05$; exploratory behaviour: Mann-Whitney Rank Sum test, $p > 0.05$). **(Bi)** Schematic illustrating the training procedure used for animals that were maintained for 7 days in either constant darkness or on the 12:12 hour normal light-dark cycle. Both groups were operantly conditioned using only two training sessions and tested for LTM after 24 hours. **(Bii)** Animals exposed to constant darkness (n = 10) exhibited LTM after only two training sessions, whereas animals exposed to the normal light-dark cycle (n = 10) did not exhibit LTM. * $p < 0.05$, ** $p < 0.01$ relative to S1. ### $p < 0.001$ relative to S2.

Figure 16Aii illustrates a significant reduction in the behaviour during the MT (normalized to S1) in the conditioned group compared to the yoked controls (Mann-Whitney Rank Sum Test, $p < 0.05$). The effects of operant conditioning were also tested using freely-behaving tests both before and after training, as done previously (Lowe and Spencer, 2006; Rothwell and Spencer, 2014) and only conditioned animals (but not yoked controls) showed a significant reduction in their pneumostome openings from the pre-test to the post-test (Conditioned: pre: 5.00 ± 0.52, post: 2.08 ± 0.23, $p < 0.001$; Yoked: pre: 5.25 ± 0.30, post: 4.92 ± 0.66, $p > 0.05$; data not shown).

Animals were next incubated in either RALDH inhibitor, or the vehicle, and randomly assigned to either constant darkness (48 hours prior to S1) or to normal 12:12 hour light-dark conditions prior to operant conditioning (Figure 16B). The open lab environments were used for all of the following experiments (unless otherwise stated). A mixed factor ANOVA revealed a significant interaction effect between condition and session ($F_{(10,186)} = 5.35$; $p < 0.001$) and *post-hoc* analysis revealed that animals incubated in EtOH (vehicle controls) and then operantly conditioned with four 45 minute sessions, demonstrated both learning and LTM 24 hours later, regardless of whether they were exposed to constant darkness (n = 17) or the normal 12:12 hour light-dark cycle (n = 16) (Figure 16Ci). These data mirror previous findings which suggest that EtOH has no effect on the ability of the animals to show learning and memory (Rothwell and Spencer, 2014). These data also confirm that 48 hours of darkness prior to training did not affect the ability of animals to form LTM following four training sessions. Animals incubated in either of the RALDH inhibitors citral (n = 16; Figure 16Cii) or DEAB (n = 16; data not shown), in the normal 12:12 hour light-dark cycle also demonstrated learning following four training sessions. As expected, based on our previous findings (Rothwell and Spencer, 2014), animals exposed to either citral or DEAB in the normal light-dark cycle did not form LTM at 24 hours (Figure 16D).

Interestingly however, animals incubated in either citral (n = 17; Figure 16Cii; 16D) or DEAB (n = 17; Figure 16D) and maintained under constant darkness prior to training, demonstrated LTM at 24 hours. Specifically, animals incubated in citral under constant darkness for 48 hours prior to S1 demonstrated a significant reduction in attempts at aerial respiration from S1 to S4 ($p < 0.001$) and maintained this significant reduction in behaviour in the MT ($p < 0.001$ relative to S1; Figure 16Cii). Animals incubated in DEAB under constant darkness also demonstrated a significant reduction in behaviour from S1 to S4 ($p < 0.001$) which persisted for 24 hours ($p < 0.001$) demonstrating LTM (Figure 16D). Yoked control animals incubated in citral under constant darkness (n = 17) did not show any significant changes in behaviour across training sessions or in the MT ($p > 0.05$; data not shown). The behaviour of the darkness-yoked control animals in the MT (normalized to S1) was not significantly different from the light-yoked control animals, but was significantly different from the light-operantly conditioned animals (Figure 16D). These data confirm that citral or darkness treatment alone did not affect respiratory behaviour.

These experiments with citral (and EtOH controls) were also repeated using the incubation boxes (enclosed environment; data not shown), and similar outcomes were produced, with a significant interaction effect revealed by a mixed factor ANOVA ($F_{(2,56)} = 10.10$; $p < 0.001$). That is, animals incubated in citral under the translucent box (normal light-dark cycle; n = 17) demonstrated learning (S1: 7.00 ± 0.59; S4:2.35 ± 0.27; $p < 0.001$) but did not form LTM lasting 24 hours (MT: 5.12 ± 0.48). However, animals incubated in citral under the black box (constant darkness; n = 17) demonstrated learning (S1:7.59 ± 0.54; S4:2.94 ± 0.38; $p < 0.001$) but now also demonstrated LTM lasting 24 hours (MT: 2.82 ± 0.29; $p < 0.001$ relative to S1). These data agree with the data collected from animals maintained in an open environment and strongly suggest that *Lymnaea* overcome the memory inhibition resulting from exposure to an

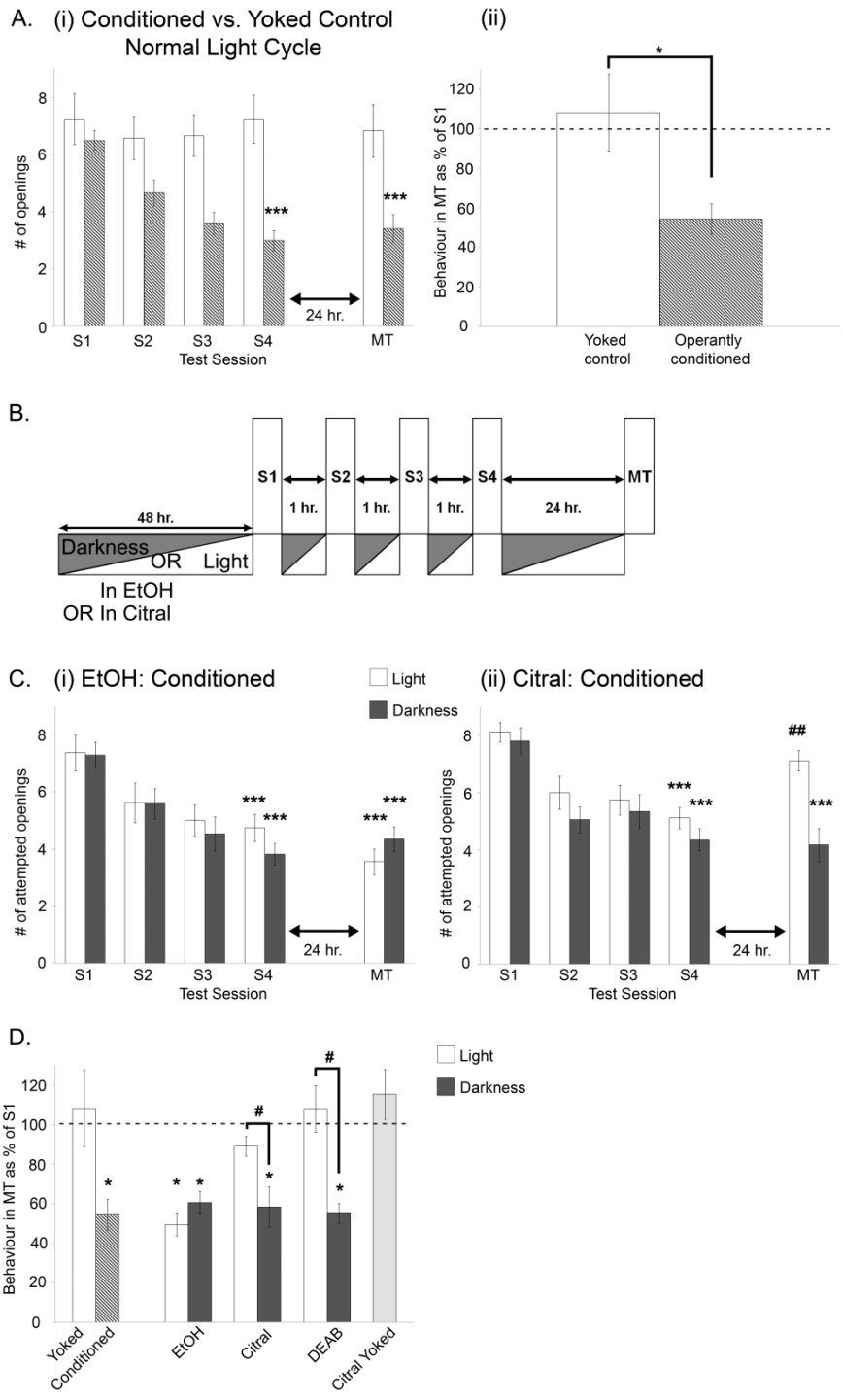
A.
(i) Conditioned vs. Yoked Control
Normal Light Cycle
# of openings
Test Session
S1
S2
S3
S4
MT
24 hr.
***
(ii)
Behaviour in MT as % of S1
Yoked control
Operantly conditioned
*
B.
48 hr.
Darkness
OR
Light
In EtOH
OR In Citral
1 hr.
24 hr.
C.
(i) EtOH: Conditioned
(ii) Citral: Conditioned
Light
Darkness
# of attempted openings
##
D.
Behaviour in MT as % of S1
Yoked Conditioned
EtOH
Citral
DEAB
Citral Yoked
#

Figure 16.

Figure 16. Citral no longer inhibits LTM formation in animals exposed to constant darkness. (Ai) Animals conditioned using four 45 minute training sessions (n = 12) showed LTM at 24 hours, whereas yoked controls (n = 12) showed no change in behaviour. (*** $p < 0.001$ relative to S1). **(Aii)** Data showing behaviour in the 24 hour MT normalized to S1 illustrate that conditioned animals show a significant reduction in behaviour compared to yoked controls. (Mann-Whitney Rank Sum test, * $p < 0.05$). **(B)** Animals were incubated in either EtOH or citral in constant darkness or in the normal 12:12 hour light-dark cycle for 48 hours before being operantly conditioned with the LTM training procedure. **(Ci)** Animals exposed to EtOH (0.35%) under both normal 12:12 hour light cycle (n = 16), as well as constant darkness (n = 17), demonstrated LTM at 24 hours following four training sessions. Animals exposed to citral in the normal light cycle **(Cii;** n = 16**)** failed to demonstrate LTM, as shown previously (Rothwell and Spencer, 2014). However, animals exposed to constant darkness and citral showed LTM formation. (*** $p < 0.001$ relative to S1; ## $p < 0.01$ relative to S4; n = 17). **(D)** Data showing behaviour in the MT normalized to that of S1 illustrate that both RALDH inhibitors, citral and DEAB, prevented LTM formation when animals were exposed to normal light cycles, but failed to inhibit LTM when the animals were exposed to constant darkness. Note that exposure to citral alone in the darkness did not affect the behaviour of yoked control animals in the MT. (* $p < 0.05$ relative to yoked control (citral treated). # $p < 0.05$; Kruskal-Wallis 1-way ANOVA on ranks; Dunn's *post-hoc* test in **D** only).

RALDH inhibitor when concurrently exposed to darkness. The RALDH inhibitors therefore no longer impair LTM formation when combined with constant exposure to darkness.

Exposure to darkness does not overcome the memory impairment induced by a retinoid receptor antagonist

We previously showed that *Lymnaea* exposed to the RXR pan-antagonist HX531 (10^{-6} M) were unable to form LTM at 24 hours following operant conditioning, whereas HX531 had no effect on the respiratory behaviour of yoked control animals (Rothwell and Spencer, 2014). Our next aim was to determine whether this retinoid receptor antagonist could continue to prevent LTM formation when the animals were simultaneously exposed to darkness. That is, could the memory-enhancing abilities of darkness again overcome the inhibitory effects of blocking retinoid signaling?

Animals were incubated in either the retinoid receptor antagonist HX531 (10^{-6} M) or 0.01% DMSO (vehicle controls) for 48 hours prior to S1 and animals from each group were exposed to constant darkness (48 hours) or normal light-dark cycles in an open lab environment. HX531 and DMSO-treated animals were then conditioned using four training sessions and given a MT 24 hours later (Figure 17A). A mixed factor ANOVA revealed a significant interaction effect between condition and session ($F_{(6,112)} = 4.38$; $p < 0.001$). *Post-hoc* analysis revealed that, as expected, animals incubated in 0.01% DMSO demonstrated learning following four training sessions, as well as LTM at 24 hours, regardless of whether they were exposed to a normal light-dark cycle (n = 15) or constant darkness (n = 15) (Figure 17B). Animals incubated in the retinoid receptor antagonist, HX531 and maintained under the normal light-dark cycle (n = 15) demonstrated a significant reduction in pneumostome openings from S1 to S4 ($p < 0.001$) and thus demonstrated learning (Figure 17C). As expected from previous studies (Rothwell and

Spencer, 2014), these animals did not, however, form LTM lasting 24 hours (Figure 17C; S4 vs. MT $p < 0.05$). Animals incubated in HX531 under constant darkness ($n = 15$) also demonstrated a significant reduction in respiratory behaviour from S1 to S4 ($p < 0.001$; Figure 17C), thus also showing learning. Interestingly, however, these animals incubated in HX531 in darkness did not form LTM at 24 hours, as the number of pneumostome openings observed in the MT was significantly greater than that seen in S4 ($p < 0.001$) but not significantly different from S1 ($p > 0.05$; Figure 17C). Thus, it appears that exposure to darkness did not overcome the memory inhibition that results from blocking the retinoid receptor.

In summary, 48 hours of darkness acted as a memory enhancer, producing LTM following conditioning with an ITM protocol only, and was able to overcome the memory-inhibiting effects of the RALDH inhibitors. However, the memory-enhancing effects of darkness did not overcome memory inhibition produced by the retinoid receptor antagonist. These data suggest that the mechanism by which darkness enhances memory is not affected by the inhibition of retinoic acid synthesis, yet is affected by downstream inhibition of signaling at the retinoid receptor.

Cyp26 mRNA levels are not affected by darkness

Following these findings, we hypothesized that constant darkness might up-regulate the retinoid signaling pathway in a manner that would lead to enhanced retinoid signaling and promotion of memory formation. The Cyp26 enzymes are responsible for retinoic acid degradation and have previously been shown to be affected by photoperiod (Helfer et al., 2012). We hypothesized that if Cyp26 levels were down-regulated in darkness, retinoic acid levels might remain higher (even during inhibition of RALDH). This might explain how darkness could overcome the effects of the RALDH inhibitors, but could not overcome the effects of the retinoid

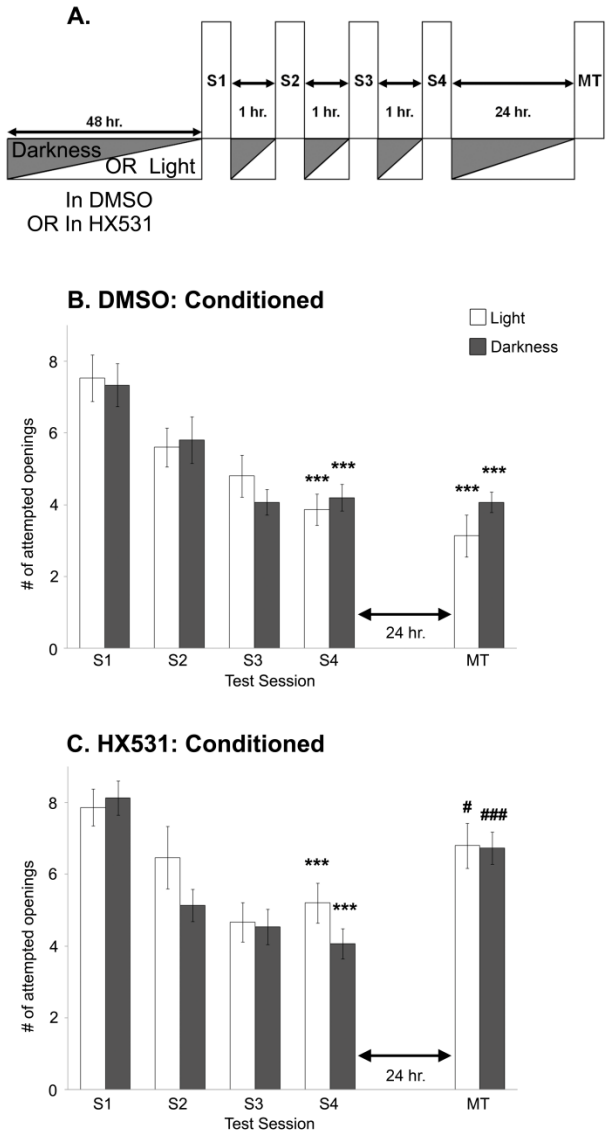

Figure 17. Exposure to the retinoid receptor antagonist HX531 inhibits LTM formation, even following constant darkness. **(A)** Animals were incubated in DMSO or HX531 for 48 hours before S1, under either constant darkness or the normal light-dark cycle. Animals were then trained with the LTM conditioning procedure. **(B)** Animals exposed to DMSO exhibited both learning and LTM at 24 hours, regardless of whether they were exposed to the normal 12:12 hour light cycle (light; n = 15) or to constant darkness (n = 15). **(C)** Animals exposed to the retinoid receptor antagonist HX531 demonstrated learning, but failed to show LTM formation at 24 hours, regardless of whether exposed to the normal 12:12 hour light cycle (light; n = 15) or constant darkness (n = 15). *** $p < 0.001$ relative to S1; # $p < 0.05$, ### $p < 0.001$ relative to S4.

receptor antagonist (which would block any downstream effects of retinoic acid).

To examine this, our next aim was to first determine whether 48 hours of constant darkness affected the mRNA levels of either *Lym*Cyp26 or *Lym*RALDH, both of which have recently been cloned from the *Lymnaea* CNS. Using quantitative real-time PCR, we first isolated CNS from *Lymnaea* that had been maintained in either 48 hours of darkness or in 12:12 hour normal light-dark cycles only (no training). There were, however, no significant differences between the relative expression levels of either *Lym*Cyp26 or *Lym*RALDH following exposure to different light/dark treatments (Figure 18A; $p > 0.05$). However, animals maintained in darkness and then trained showed higher levels of *Lym*RALDH and lower levels of *Lym*Cyp26 than untrained animals in the darkness (data not shown). Though this analysis suggested that training itself might affect retinoid signaling pathways (likely resulting in higher retinoic acid levels), it did not itself explain how training following darkness enhanced memory formation compared to training following normal light-dark cycles. Thus, our next aim was to determine whether the specific combination of darkness and training had a significant effect on retinoid enzyme mRNA levels. Animals were maintained in either constant darkness or the normal 12:12 hour light-dark cycle for 48 hours. All animals were then given two training sessions and a memory test 24 hours later, after which, the CNS were immediately isolated and frozen. Real-time qPCR showed that the mRNA levels of *Lym*RALDH were higher in the darkness group compared to the normal light-dark cycle group, though the increase did not reach a significant level (Figure 18Bi). The relative expression levels of Cyp26 were unchanged (Figure 18Bii). We also examined the levels of the retinoid receptors, *Lym*RXR and the putative *Lym*RAR in the two groups of animals, but found no significant differences (RXR: $p = 0.28$; RAR; $p = 0.97$; data not shown).

In summary, exposure to constant darkness for 48 hours, in the absence of training, had no apparent effect on the mRNA levels of the retinoid metabolic enzymes. However, the

combination of darkness and training together did have a small, albeit non-significant, effect on RALDH mRNA levels. Though these findings did not support our initial hypothesis that exposure to darkness might result in enhanced retinoid signaling as a result of reduced Cyp26 levels, it does provide preliminary support for a combined effect of darkness and conditioning on retinoid signaling pathways.

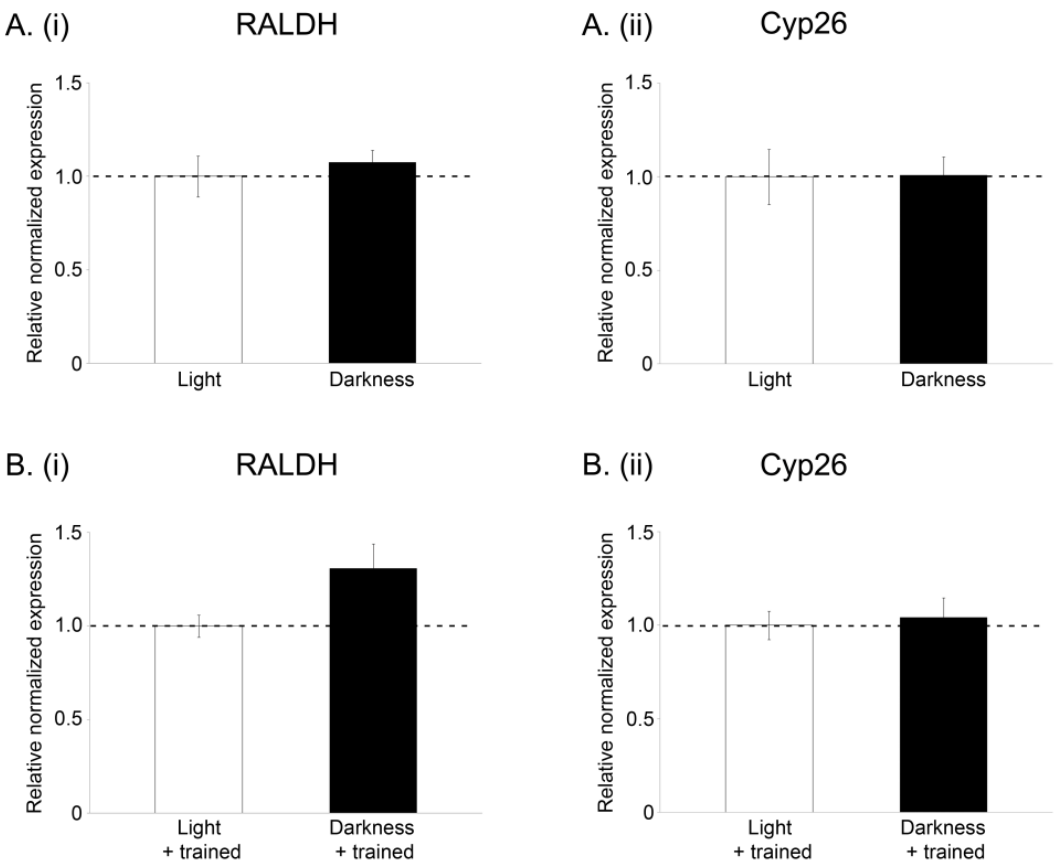

Figure 18. Training and darkness showed no significant changes in RALDH or Cyp26 mRNA levels in the CNS, compared to normal light conditions. Animals were exposed to either the normal 12:12 hour light cycle or constant darkness for 48 hours, after which time the CNS were dissected for RNA extraction. Data are expressed as mean relative normalized expression ± SEM and show no significant difference in the levels of either *Lym*RALDH **(Ai)** or *Lym*Cyp26 **(Aii)** (with β-tubulin, EIF4α, 18srRNA, and actin used as normalizer genes). However, if after 48 hours of light/dark treatments, the animals were then trained with two 45 minute training sessions and the CNS dissected immediately after the MT, the relative normalized expression levels of *Lym*RALDH **(Bi)** became elevated (albeit not to a significant level; $p = 0.102$). There was no significant change in the relative expression levels of Cyp26 **(Bii)**. β-tubulin, EIF4α, and actin were used as normalizer genes.

3.05 Discussion

We have previously shown that LTM formation following operant conditioning of the aerial respiratory behaviour was prevented following disruption of retinoid signaling (Rothwell and Spencer, 2014). These previous findings mirror those from vertebrates and reveal the importance of the retinoid signaling pathway in invertebrate implicit memory, in addition to vertebrate hippocampal-dependent memory (Chiang et al., 1998; Misner et al., 2001). We had shown previously that incubation of animals in various retinoid receptor agonists also promoted the formation of LTM using a training paradigm that normally produced only ITM. A serendipitous discovery that exposure of animals to constant darkness had similar memory-promoting abilities as the retinoid receptor agonists (converting ITM into LTM), led us to propose that exposure to constant darkness might be enhancing retinoid signaling within the animal. We thus set out to determine how combining the effects of constant darkness with various retinoid signaling inhibitors might affect memory formation; in particular, whether the memory-promoting effects of darkness would override the memory inhibition produced by inhibiting retinoid signaling. Interestingly, we found that the RALDH inhibitors citral and DEAB were no longer able to prevent long-term memory formation in animals exposed to constant darkness. However, the retinoid receptor antagonist HX531 continued to inhibit long-term memory (consistent with previous findings), regardless of whether the animals were exposed to darkness or not. These results suggest that darkness might somehow affect the strength of the existing retinoid signaling pathway, as it was able to overcome the effects of blocking production of retinoic acid, but was unable to overcome the effects of blocking downstream retinoic acid signaling via the retinoid receptor. Exactly how darkness and retinoid signaling interact, however, has not yet been determined.

Many studies have investigated the effects of circadian rhythms on learning and memory formation in different species such as mice, zebrafish and molluscs (reviewed in Lyons, 2011). For example, the diurnal mollusc *Aplysia* shows robust long-term memory when trained in the day, but little memory if trained at night (following either a non-associative sensitization paradigm (Fernandez et al., 2003) or an operant conditioning paradigm (Lyons et al., 2005)). These studies often used constant darkness conditions prior to investigating memory formation during the subjective day or night. Although Lyons et al. (2005) did not directly compare the memory obtained from animals following exposure to darkness versus light-dark cycles (and thus did not examine any possible memory-enhancing effects of darkness), their data suggest that some memory scores may have improved at specific test times following exposure to darkness. However, it should also be noted that in many of the previous studies, memory was tested at a time when LTM was known to be formed, and so any memory-enhancing effects of darkness would not be observed. Likewise, in our study, when we trained animals with the normal LTM protocol (4 sessions), there was no apparent memory-enhancing effects of darkness observed. It was only when we trained animals with the ITM procedure (2 sessions), but tested for LTM, that the memory-enhancing effects of constant darkness became apparent. It should be noted that all training and testing of our animals (regardless of the pharmacological treatment) was conducted during the same time of day and under normal light conditions, and so any normal circadian effects on memory formation were not responsible for the observed effects.

Diurnal zebrafish trained with an avoidance operant conditioning paradigm also show better learning and 24 hour retention when trained and tested during the day than during the night (Rawashdeh et al., 2007). Experiments further showed that this was likely a result of melatonin, the higher levels of which, at night, led to memory impairment. These studies also used animals maintained in constant darkness (for up to 3 to 4 days). Once again, these authors did not directly

compare the effects of constant darkness with normal light-dark cycles, but their data suggest that the retention scores, when measured at the same time of day, were lower in the group maintained in constant darkness than those maintained on a light-dark cycle. These results are opposite to those observed in our study in which retention was improved by constant darkness (when measured at the same time of day as normal light-dark cycle animals). However, any potential for darkness to enhance memory in the zebrafish may have been confounded by the particular training paradigm used in that study, where animals received mild electric shocks in the dark regions of the tank.

Interestingly, melatonin has also been linked to changes in retinoid signaling in rats (Helfer et al., 2012). Three days of melatonin administration caused a significant reduction of the Raldh1 transcript, though Cyp26B1 was unaffected by the 3 day treatment and was only reduced after 14 days. Three days of induced photoperiod change was also sufficient to induce changes in the expression of both Raldh1 and Cyp26B1. Indeed, many studies conducted in rats and mice have now shown that retinoic acid synthesizing and transducing components are regulated by photoperiod. However, they have shown that a reduction of photoperiod (from 16 hours to 8 hours of light) generally causes a reduction in retinoic acid signaling, by down-regulating RALDH, Cyp26 and the retinoid receptors. Conditions of longer light duration thus increase the strength of the retinoic acid signal (Shearer et al., 2010). These results are perhaps opposite to what we might predict from our study, where we would hypothesize that the memory promoting effects of darkness might result from enhanced retinoic acid signaling. However, it should be noted that rodents are generally nocturnal species, whereas *Lymnaea* are more active during the day. Indeed, even amongst rodents, species differences in the retinoid signaling response to photoperiod can occur; for example, RXRγ levels are down-regulated in hamsters during shorter photoperiods, but are up-regulated in rats (Ross et al., 2004; Helfer et al., 2012).

Photoperiod-induced changes in retinoid signaling observed in rats and mice occur in the hypothalamus and are likely linked to changes in food intake and weight and thus accompany physiological changes that might occur during seasonal changes. Such photoperiod-induced changes in retinoic acid signaling are thus likely very different from a sudden transition to constant darkness, as performed in our study. However, the fact that retinoic acid signaling can indeed be affected by light-dark cycles and/or by melatonin in vertebrates, certainly suggests that light cycles and retinoid signaling may also be linked in some way in *Lymnaea*. Our initial attempt to determine if this might be the case was to first determine whether 48 hours of constant darkness might alter the mRNA levels of enzymes involved in retinoic acid metabolism. Using real-time quantitative PCR, we found that there were no significant differences in the relative mRNA levels of either *Lym*RALDH or *Lym*Cyp26, based on the light/dark conditions; 48 hours of constant darkness alone, had no apparent effect. Interestingly, however, when darkness was combined with the aversive operant conditioning paradigm, the mRNA levels of RALDH rose (albeit non-significantly), though there were no changes in either *Lym*Cyp26, or in the retinoid receptor mRNA levels (*Lym*RXR and *Lym*RAR). How an increased expression of RALDH alone might have resulted in overriding the memory disruption produced by the RALDH inhibitors is, however, unclear. One might speculate that a small increase in mRNA levels might produce larger changes in protein levels, possibly overwhelming the inhibitors used. However, we can only speculate at this time, due to the lack of functioning antibodies for *Lym*RALDH. It is also not currently possible to measure how retinoic acid levels might change under varying conditions of darkness and training, as over 75 CNS were previously required merely to detect retinoic acid in the *Lymnaea* CNS (Dmetrichuk et al., 2008). It is also feasible that the levels of other retinoid signaling molecules may have been altered by constant darkness (for example, cellular retinoic acid binding proteins), but as no other retinoid signaling molecules have currently been identified or cloned in *Lymnaea*, we were not able to examine this possibility.

Though we have speculated that constant darkness might have direct effects on retinoid metabolism and/or retinoid signaling, we also cannot rule out the possibility that darkness promoted memory formation by another means, or by other pathways that converge downstream with the retinoid pathway. It has previously been shown that exposure to stressors, such as predator detection or acute exposure to KCl, can promote memory formation following operant conditioning of the respiratory behaviour in *Lymnaea* (Orr and Lukowiak, 2008; Lukowiak et al., 2008; Orr et al., 2009; Dalesman et al., 2011a). Specific stress hormones have not been fully characterized in *Lymnaea* (though an ACTH-like molecule (Sonetti et al., 2005) and serotonin (Lukowiak et al., 2008) are possible candidates), so it was not possible to directly test stress hormone levels. Instead, we tested whether constant darkness affected two behaviours (righting response and exploratory behaviour) that are known to be significantly affected by stress (Orr et al., 2007). We also examined whether the animals would eventually habituate to the stress if given a longer incubation in darkness. Overall, none of our findings suggested that darkness was acting merely as a stressor on the animals to promote memory formation.

In summary, we have shown here that 48 hours of constant darkness enhanced memory formation following aversive operant conditioning in *Lymnaea*. These memory-enhancing effects of darkness were capable of overriding the memory-inhibiting effects of RALDH inhibition, but were not capable of overriding the effects of retinoid receptor inhibition. These data provide further insights into the importance of environmental light/dark conditions in molluscan memory formation, as well as how these might interact with other factors affecting memory formation, such as retinoid signaling.

3.06 Acknowledgements

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Chapter 4:

The role of retinoic acid in synapse formation and modulation of cultured *Lymnaea* neurons

4.01 Abstract

Retinoid signaling is involved in the formation of hippocampal-dependent memories in vertebrates, and affects the underlying synaptic plasticity in the form of long-term potentiation (LTP) and long-term depression (LTD). Additionally, RA rapidly modulates transmission at both electrical and chemical synapses in vertebrates. Various trophic factors have been shown to influence synaptic plasticity and RA can exert a trophic influence, at least to support and enhance neurite outgrowth (in both vertebrates and invertebrates). Having previously shown that RA signaling is important for LTM formation in *Lymnaea*, I next examined whether RA might act as a trophic factor to influence synaptogenesis and/or synaptic transmission between identified molluscan neurons in culture. By nature of my original experimental design, I could not determine whether RA alone was sufficient to promote excitatory synaptogenesis because, contrary to previously published reports, excitatory synapses formed in the absence of exogenous trophic factors. However, it appeared that RA may influence the strength of excitatory synapses formed in culture. I also examined the effects of RA on synaptic plasticity, but found no evidence to indicate that RA influenced post-tetanic potentiation of a chemical synapse. Retinoic acid did, however, reduce transmission at electrical synapses in a cell-specific manner. This was the first investigation into whether RA influences synapse formation and/or plasticity in an invertebrate species and this study provides preliminary evidence that RA may influence electrical synapses between central neurons within the *Lymnaea* CNS.

4.02 Introduction

Retinoic acid (RA) is active in both the developing and adult CNS (Maden, 2002, 2007). In the adult vertebrate nervous system, disruptions in retinoid signaling impaired hippocampal-dependent memory formation (Chiang et al., 1998; Wietryzch et al., 2005). I have now demonstrated that RA is also required for the formation of implicit memory in the mollusc *Lymnaea stagnalis*. Specifically, inhibiting retinoid signaling prevented LTM formation, while activation of retinoid receptors promoted LTM formation in this mollusc (Chapter 2). It has been widely shown that memory formation requires both changes in the strength of existing synapses as well as the formation of new synapses. Long-term potentiation (LTP; increased synaptic strength) and long-term depression (LTD; reduced synaptic strength) are two forms of hippocampal synaptic plasticity which underlie memory formation (Kandel et al., 2014), and both are influenced by RA. Vitamin A deficient rodents demonstrate impairments in both LTP and LTD (Misner et al., 2001), while retinoid receptor mutant mice also show impaired LTP (Chiang et al., 1998; Nomoto et al., 2012). Retinoic acid also controls the translation of glutamate receptor 1 within hippocampal dendrites (Poon and Chen, 2008) and increases dendritic growth within hippocampal neurons (Chen and Napoli, 2007). Thus, there is evidence that RA is involved in cellular and molecular events underlying synaptic plasticity in the vertebrate nervous system.

Trophic factors are known to play an important role in both synapse formation and synaptic modulation, and various trophic factors, including, but not limited to, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) influence hippocampal LTP (Yamada et al., 2002; Shimazu et al., 2006). Retinoic acid is known to act as a trophic factor to support neuronal survival and outgrowth in both vertebrates (e.g. embryonic rat spinal cord tissue; Wuarin et al., 1990) and invertebrates (e.g. cultured adult *Lymnaea* neurons; Dmetrichuk et al.,

2006, 2008; Carter et al., 2010). Additionally, RA affects transmission at both electrical (Weiler et al., 1999; Zhang and McMahon, 2000) and chemical (Aoto et al., 2008; Sarti et al., 2013) synapses in vertebrates. However, whether RA affects synaptic plasticity in an invertebrate species has not yet been determined. Furthermore, whether RA exerts trophic support for synaptogenesis has not been studied in either vertebrates or invertebrates. One likely reason for this is that synapse formation usually follows neurite outgrowth, which RA is known to affect. The *Lymnaea* CNS offers the advantage of examining synapse formation in the absence of neurite outgrowth, by culturing neurons in a soma-soma configuration (Feng et al., 1997; Hamakawa et al., 1999; Woodin et al., 1999). Using this approach, it has been shown that both inhibitory and excitatory synapses will reform in culture. However, it has been shown that only excitatory synapses require extrinsic trophic factors (Hamakawa et al., 1999; Woodin et al., 1999), while inhibitory synapses do not (Feng et al., 1997).

Having shown that retinoid signaling is required for LTM formation following operant conditioning in *Lymnaea*, I next hypothesized that this may be because RA influences either synaptogenesis or synaptic plasticity. Thus, I examined identified pre- and post-synaptic neurons in culture to determine whether RA can exert a trophic influence to either support excitatory synaptogenesis, and/or regulate synaptic transmission at chemical or electrical synapses.

4.03 Materials and Methods

Animals

Lymnaea stagnalis (originally obtained from stocks at the Vrije University, Amsterdam) were bred in the laboratory environment and maintained at room temperature on a fixed 12:12 hour light-dark cycle. *Lymnaea* were fed a combination of NutraFin Max Spirulina fish food (Hagen) and romaine lettuce. All animals used for cell culture ranged in shell length from 15 to 20 mm.

Chemicals

All-*trans* retinoic acid (atRA) was purchased from Sigma-Aldrich and prepared in 100 % EtOH at a stock concentration of 10^{-2} M. All-*trans* RA was then diluted in defined medium (DM; 50 % Leibovitz's L-15 medium, Gibco) to the final desired concentration (as specified for each experiment). Stock solutions of atRA were made fresh daily.

Cell Culture

All dissections were conducted under sterile conditions, as previously described (Dmetrichuk et al., 2006; Vesprini and Spencer, 2014). Animals were anaesthetized by submersion in normal *Lymnaea* saline containing 25 % Listerine® and then were placed in a dissection dish containing antibiotic *Lymnaea* saline (ABS; normal *Lymnaea* saline containing 225 µg/mL gentamicin (Sigma-Aldrich)). *Lymnaea* were dissected to expose the central ring ganglia (CNS), and the CNS was then removed and given three 10 to 15 minute washes in ABS. The CNS were next treated with trypsin (Sigma-Aldrich; 2 mg/mL in DM) for 19 to 22 minutes at 21°C, followed by treatment with trypsin inhibitor (Sigma-Aldrich; 2 mg/mL in DM) for 10

minutes. The CNS were pinned out in high osmolarity DM and the outer sheath of connective tissue was removed, followed by the subsequent removal of the inner sheath.

Individual identified neurons were removed from the ganglia with a fire polished, Sigmacoted (Sigmacote; Sigma-Aldrich) glass pipette using suction applied via a microsyringe. Cells were plated on poly-L-lysine (Sigma-Aldrich) coated culture dishes containing 3 mL of DM. The culture substrate was either poly-L-lysine-coated plastic or glass depending on the specific experiment. The somata of identified neurons were juxtaposed within the culture dish in order to facilitate the formation of soma-soma synapses, and cells were kept in the dark at room temperature overnight (at least 18 hours). For some experiments, atRA or EtOH were added to the dish at the time of culture for a final bath concentration of 10^{-7} M atRA or 0.001 % EtOH (vehicle control).

Three different cell pairs which have been previously examined both *in vivo* and *in vitro* were used to examine synaptogenesis and synaptic transmission. The same presynaptic neuron (Visceral Dorsal 4; VD4) was used in all experiments, but the postsynaptic partner changed depending on the nature of the experiment. The postsynaptic target cells used in this study were Left Pedal Dorsal 1 (LPeD1), Right Pedal Dorsal 1 (RPeD1), and Right Pedal Dorsal 2 (RPeD2).

Electrophysiology

Standard intracellular recording techniques were used to make simultaneous electrophysiological recordings from soma-soma cell pairs at least 18 hours after the time of culture in order to examine synapse formation. Glass electrodes (30 to 60 MΩ in resistance) were pulled on a Kopf vertical pipette puller (Model 730, David Kopf Instruments) and filled with saturated potassium sulphate. An intracellular recording amplifier (NeuroData IR283A; Cygnus

Technology Inc.) and a Powerlab 4sp data acquisition system (with Chart v4.2.4 software; AD Instruments) were used for all electrophysiological recordings.

A. All-trans RA and excitatory synaptogenesis

An excitatory synapse exists from VD4 to LPeD1 *in vivo*, and this synapse has previously been reformed in the soma-soma configuration in cell culture (Hamakawa et al., 1999). Thus, I used this cell pair to examine whether RA influences excitatory synaptogenesis. VD4 and LPeD1 were paired in a soma-soma configuration on plastic or glass substrates coated with poly-L-lysine in either i) DM alone, ii) 10^{-7} M atRA, or iii) 0.001 % EtOH (vehicle control). This concentration of atRA has been shown to be effective in inducing neurite outgrowth from cultured *Lymnaea* neurons (Dmetrichuk et al., 2006). Synapses were classified as either chemical (excitatory or inhibitory), electrical, or a combination of chemical and electrical (i.e. 'mixed'). For analysis of excitatory postsynaptic potentials (EPSPs) recorded in LPeD1, the amplitude of at least 3 EPSPs was measured during the first 15 minutes of a recording to avoid any effects of synaptic rundown. An electrical component was deemed to be present when the coupling coefficient was greater than 5 % (as this produced sufficient deflection in the postsynaptic cell such that electrical coupling could be easily distinguished from any deflection resulting from noise or possible 'cross-talk' between the electrodes). The membrane potential of LPeD1 was maintained constant throughout each recording.

The presence or absence of an appropriate excitatory synapse between VD4 and LPeD1 was compared between culture conditions using Fisher's Exact tests which were corrected using the Bonferroni-Holm step-down method. The amplitude of the EPSP response was expressed as mean ± SEM. For the cell pairs in which an excitatory synapse formed on the plastic substrate,

the amplitude of the EPSP observed in LPeD1 was compared across culture conditions using a one-way ANOVA.

B. All-trans RA and post-tetanic potentiation

VD4 and RPeD1 were paired in a soma-soma configuration and cultured in DM overnight. The resting membrane potential of the postsynaptic cell, RPeD1, was held constant throughout each recording to prevent changes in the driving force that might otherwise alter the size of the postsynaptic responses. Single control EPSPs were generated in RPeD1 as a result of one action potential in VD4 elicited by injection of depolarizing current. The amount of depolarizing current used to generate one action potential in VD4 was constant throughout each trial. At least three to four control EPSPs (spaced at least three seconds apart) were generated for each test of post-tetanic potentiation, and their amplitudes were averaged during analysis. A constant amount of depolarizing current was then injected into VD4 for a constant duration, to induce a train of action potentials (tetanus) to generate a complex depolarized response in RPeD1. A constant amount of depolarizing current was then injected into VD4 to elicit a single action potential within 10 seconds after the tetanic activation of VD4. This elicited the potentiated EPSP in RPeD1. The presence of post-tetanic potentiation at the synapse from VD4 to RPeD1 was verified at baseline (prior to the application of EtOH or atRA) using paired t-tests to compare the amplitude of the control and potentiated EPSPs. All-*trans* RA or EtOH were then added to the bath solution following initial baseline recordings to produce a final bath concentration of 10^{-6} M atRA (Aoto et al., 2008) or 0.01 % EtOH. Synaptic efficacy and post-tetanic potentiation were examined approximately every five minutes over the next 60 minutes. The maintenance of post-tetanic potentiation during exposure to EtOH or atRA was also tested using paired t-tests by analyzing data at 10, 30, and 60 minutes together in each condition. The

effect of EtOH and atRA on post-tetanic potentiation over time was analyzed by expressing the amplitude of the potentiated EPSP as a percentage of the amplitude of the control EPSP $\left(\left(\frac{potentiated\ EPSP\ amplitude}{control\ EPSP\ amplitude}\right) \times 100\right)$. A mixed factor ANOVA with drug condition as the fixed factor and time as the repeated factor was used to compare post-tetanic potentiation over time. A Tukey *post-hoc* test was used for comparisons, which were deemed significant when $p < 0.05$. The maximum depolarization of RPeD1 resulting from tetanic stimulation of VD4 (within 20 action potentials in VD4) was also measured over time and analyzed using a mixed factor ANOVA with drug condition as the fixed factor and time as the repeated factor. A Tukey *post-hoc* test was used for comparisons, which were considered significant when $p < 0.05$. All values are presented as mean ± SEM unless otherwise stated.

C. All-trans RA and electrical synapses

The influence of RA on synaptic transmission at electrical synapses was examined using two different cell pairs in culture: i) VD4 and RPeD2 and ii) VD4 and LPeD1. While these cell pairs do not normally form electrical connections, it has been shown that electrical synapse formation can precede the formation of chemical synapses (Todd et al., 2010). Thus, the synapses examined in this study may have preceded the formation of chemical synapses which exist between these cells *in vivo*. The presence or absence of an electrical synapse was tested by injecting hyperpolarizing current into the presynaptic neuron and measuring the postsynaptic response. Either atRA or EtOH was added to the bath following baseline recordings to produce a final bath concentration of 10^{-5} M RA (Zhang and McMahon, 2000) or 0.1 % EtOH, and recordings were made for an additional 60 minutes. The baseline coupling coefficient was calculated prior to the addition of RA (or EtOH) and compared to that at 10, 30, and 60 minutes

following addition. Electrical coupling was bidirectional (unless otherwise stated), and thus the coupling coefficient was measured in both directions over time. For VD4/RPeD2 cell pairs a mixed factor ANOVA with drug condition as the fixed factor and time as the repeated factor was used to compare electrical coupling over time. A Tukey *post-hoc* test was used for comparisons, which were deemed significant when $p < 0.05$. For VD4/LPeD1 pairs a one-way RM ANOVA was used to examine changes in electrical coupling over time for each condition. Electrical coupling at 60 minutes was compared between treatments using a t-test (for coupling from LPeD1 to VD4) and a Mann-Whitney Rank Sum Test (for coupling from VD4 to LPeD1). All values are presented as mean ± SEM unless otherwise stated.

4.04 Results

Excitatory synapses form in the absence of extrinsic trophic factors

Previous research studying soma-soma synapses formed between isolated *Lymnaea* neurons demonstrate that the formation of excitatory synapses between VD4 and LPeD1 in culture required the presence of extrinsic trophic factors (Hamakawa et al., 1999; Woodin et al., 1999). Since RA exerts a trophic influence to induce neurite outgrowth in vertebrates and invertebrates (Wuarin et al., 1990; Dmetrichuk et al., 2006, 2008; Carter et al., 2010), my first aim was to examine whether RA could exert a trophic influence to support excitatory synaptogenesis. To this end, the same cell pair used in previous studies (VD4 and LPeD1) was used in this study.

Cell pairs were cultured on poly-L-lysine coated plastic culture dishes in 10^{-7} M atRA, 0.001 % EtOH (vehicle control), or DM alone and intracellular electrophysiological recordings were used to assess the presence or absence of an excitatory synapse from VD4 to LPeD1. Excitatory synapses formed in 8 of the 9 cell pairs cultured in the presence of 10^{-7} M atRA while an electrical synapse formed between the remaining cell pair. However, unexpectedly, excitatory synapses also formed between VD4 and LPeD1 in both control conditions (DM alone and 0.001 % EtOH), albeit in fewer cell pairs than in RA (Table 1; Figure 19). An excitatory synapse formed in 3 of 9 pairs cultured in DM alone and 5 of 10 pairs cultured in EtOH. The proportion of excitatory synapses formed in the presence of atRA was not significantly different from that in either control condition ($p > 0.05$). Interestingly, mixed excitatory and electrical synapses were observed in DM and EtOH, but not in the presence of atRA (Table 1; Figure 20A). Since electrical synapse formation can precede the formation of chemical synapses (Todd et al., 2010), this result may suggest that RA accelerated excitatory synapse formation, as electrical components to the chemical synapses were never observed in the presence of atRA.

Table 1. Excitatory synaptogenesis occurs in the absence of trophic factors. Excitatory synapses were recorded from VD4 to LPeD1 in all three culture conditions when cells were plated on a poly-L-lysine coated plastic substrate. The n values represent the number of cell pairs in each culture condition.

Culture condition	No synapse	Excitatory only	Excitatory + Electrical	Electrical only
Defined medium (DM; **n = 9**)	2	3	2	2
0.001% EtOH in DM (**n = 10**)	2	5	2	1
10^{-7} M atRA in DM (**n = 9**)	0	8	0	1

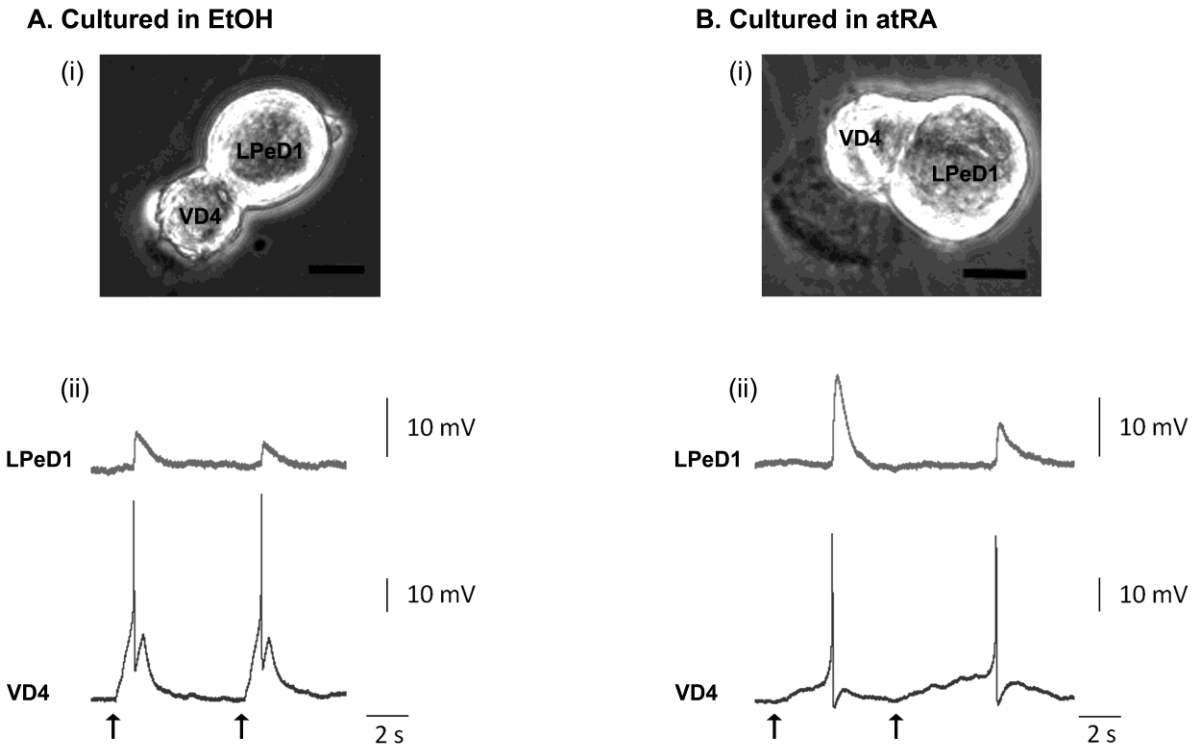

Figure 19. Excitatory synapses formed in the absence of atRA. Representative images of VD4 and LPeD1 cultured in 0.001 % EtOH **(Ai)** or 10^{-7} M atRA **(Bi)** on a poly-L-lysine coated plastic substrate. Scale bar in Ai and Bi = 30 µm. Excitatory synapses were observed in both the vehicle control **(Aii)** and atRA **(Bii)**. Arrows in Aii and Bii indicate depolarizing current injection into VD4 to invoke an action potential.

One possible explanation for my novel finding of the presence of excitatory synapses in the absence of trophic factors is that previously published studies (Hamakawa et al., 1999; Woodin et al., 1999) used a poly-L-lysine-coated glass substrate instead of the plastic I used here. My studies were thus repeated using poly-L-lysine-coated glass as the substrate, instead of plastic. However, excitatory synapses were again recorded between VD4 and LPeD1 cell pairs in the absence of extrinsic trophic factors, in both DM (3 of 6 pairs) and EtOH (2 of 7 pairs) (Table 2). Furthermore, the proportion of excitatory chemical synapses formed did not appear to be influenced by the presence of atRA (2 of 7 pairs; $p > 0.05$). One difference found on the glass substrate, compared to the plastic substrate, was that inappropriate inhibitory synapses also formed (Table 2; Figure 20B). However, these inhibitory synapses were recorded only in DM and EtOH, but not in atRA. Despite this interesting observation, the number of times these inhibitory synapses were recorded (DM = 1; EtOH = 2) were too low to conclude that atRA was preventing the formation of inappropriate synapses.

The formation of excitatory synapses in the absence of any trophic factors (in DM alone) made it impossible to determine whether RA itself exerts a trophic influence to support synaptogenesis. However, I next determined whether RA enhanced the strength of the excitatory synapses formed in culture. The amplitude of the excitatory postsynaptic potential (EPSP) was measured in LPeD1 for each excitatory synapse. An examination of the cell pairs cultured on the plastic substrate showed that the EPSP amplitude recorded in the presence of atRA was, on average, larger than that recorded in EtOH or DM, though this difference did not reach significance (atRA: 8.69 ± 1.52 mV, $n = 8$; DM: 6.85 ± 3.43, $n = 3$; EtOH: 5.47 ± 1.67 mV, $n = 5$; $p > 0.05$; Figure 21). The EPSPs recorded from cell pairs on the glass substrate were not analyzed due to the extremely low number of chemical synapses produced.

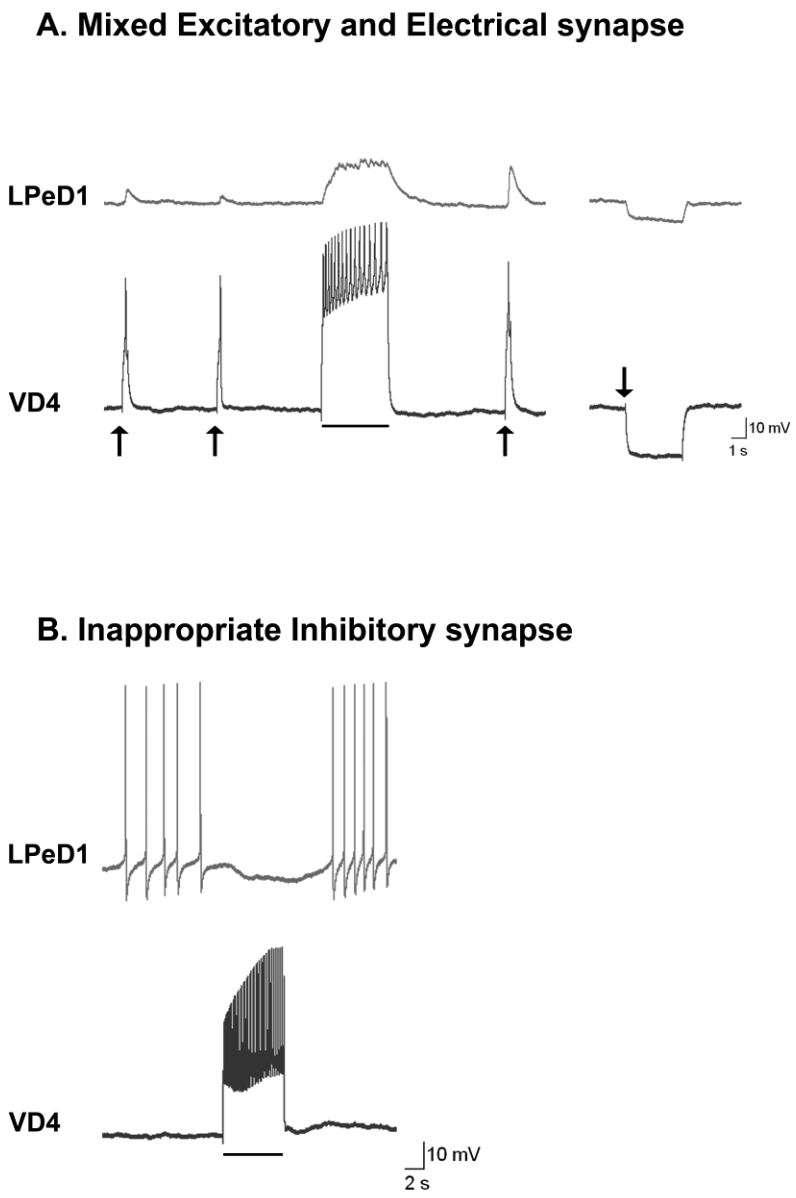

Figure 20. Both mixed electrical and chemical and inappropriate inhibitory synapses formed in the absence of atRA. (A) A mixed excitatory and electrical synapse recorded in DM on a poly-L-lysine coated plastic surface. **(B)** An inappropriate inhibitory synapse which formed in EtOH on a poly-L-lysine coated glass surface. Inappropriate inhibitory synapses were never observed in the presence of atRA. Arrows indicate the injection of current into VD4. Bars denote the duration of a depolarizing current injection to produce a train of action potentials in VD4.

Table 2. Synapse formation is more variable on a glass culture substrate than a plastic substrate. Excitatory synaptogenesis again occurred between VD4 and LPeD1 in all three culture conditions. The n values represent the number of cell pairs cultured in each condition.

Culture condition	No synapse	Excitatory only	Electrical only	Excitatory + Electrical	Inappropriate Inhibitory
Defined medium (DM; **n = 6**)	0	3	2	0	1
0.001% EtOH in DM (**n = 7**)	1	2	2	0	2
10^{-7} M atRA in DM (**n = 7**)	1	2	2	2	0

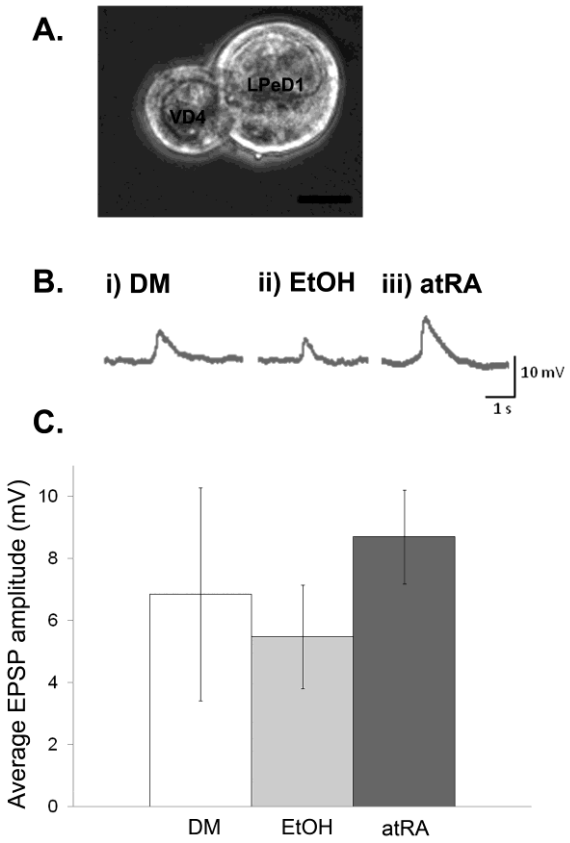

Figure 21. Excitatory postsynaptic potentials are on average larger at synapses formed in atRA than EtOH. (A) Representative image of VD4 and LPeD1 cultured in a soma-soma configuration on a plastic substrate. Scale bar = 30 µm. **(B)** Representative recordings of EPSPs in LPeD1 in excitatory synapses formed between VD4 and LPeD1 on a plastic substrate. **(C)** The excitatory synapses formed in the presence of atRA produced, on average, larger EPSP amplitudes than those formed in the presence of EtOH (vehicle) and DM, though this difference did not reach significance ($p = 0.46$).

Retinoic acid does not affect post-tetanic potentiation of a chemical synapse

Memory formation may require short-term changes in synaptic strength, and RA is known to regulate homeostatic plasticity in a rapid and non-genomic manner at vertebrate hippocampal chemical synapses (Aoto et al., 2008; Sarti et al., 2013). Post-tetanic potentiation is a form of synaptic plasticity that lasts for seconds to minutes and results in increased synaptic strength as a result of a short burst of pre-synaptic activity (Nicholls et al., 1992). Post-tetanic potentiation has previously been studied in cultured *Lymnaea* neurons (Luk et al., 2011). My next aim was to determine whether RA would enhance post-tetanic potentiation between two CPG neurons involved in the aerial respiratory behaviour of *Lymnaea*.

VD4 and RPeD1 were cultured in a soma-soma configuration, and the presence of a chemical synapse from VD4 to RPeD1, as well as the presence of post-tetanic potentiation, was confirmed during baseline recordings, prior to the addition of EtOH or atRA. A representative example of the post-tetanic potentiation recorded between VD4 and RPeD1 is shown in Figure 22. Control EPSPs were generated in RPeD1 as a result of single action potentials induced in VD4 following the injection of depolarizing current. Next, a train of action potentials was induced in VD4 via the injection of depolarizing current, and the potentiated (post-tetanic) EPSP was then generated by another single action potential in VD4. Following the demonstration of post-tetanic potentiation in a cell pair, either EtOH or atRA was added to the bathing medium to produce a final bath concentration of 10^{-6} M atRA or 0.01 % EtOH. Synaptic responses and post-tetanic potentiation were recorded for a further 60 minutes.

First, I examined whether the overall efficacy of this chemical synapse changed over time following application of atRA or EtOH (Figure 23A). The amplitudes of three to four control EPSPs produced in RPeD1 as a result of single action potentials generated in VD4 were measured and averaged at each time point. A mixed factor ANOVA examining the amplitude of

the control EPSP revealed a significant effect of time ($F_{(3,27)} = 11.492$, $p < 0.001$), but no significant interaction between time and condition. The amplitude of the control EPSP was significantly reduced over time, but there was no significant difference between EPSPs generated in the presence of either EtOH or atRA. These data suggest an overall reduction in the efficacy of the chemical synapse with time, regardless of drug condition. Thus, atRA did not prevent "rundown" of synaptic efficacy. I also determined how the potentiated EPSP amplitude changed over time in EtOH or atRA. A mixed factor ANOVA again revealed a significant effect of time ($F_{(3, 27)} = 15.342$, $p < 0.001$) and the amplitude of the potentiated EPSP was significantly reduced following the addition of both EtOH and atRA (Figure 23B). However, the ANOVA did not reveal a significant effect of condition, indicating that the amplitude of the potentiated response also decayed over time regardless of drug application.

The above data indicated that over time there was a significant decrease in the size of both the control and potentiated EPSPs when they were analyzed as separate entities. However, synaptic potentiation is measured and expressed as the size of the potentiated post-tetanic response relative to the control response. My aim was to determine whether atRA influences post-tetanic potentiation between VD4 and RPeD1. I assessed post-tetanic potentiation by expressing the amplitude of the potentiated EPSP as a percentage of the amplitude of the control EPSP. Prior to the application of atRA or EtOH, I first determined that post-tetanic potentiation had occurred at the chemical synapse between VD4 and RPeD1. Prior to the addition of atRA, a significant increase in EPSP amplitude was observed in the post-tetanic EPSP relative to the control EPSP ($p = 0.025$). Prior to the addition of EtOH, the difference between the amplitude of the potentiated EPSP and the control EPSP approached significance ($p = 0.054$) (Figure 24A). Once I had determined that post-tetanic potentiation had occurred at a synapse, atRA or EtOH were added to the bathing medium and recordings were made for another 60 minutes. Post-

tetanic potentiation was maintained in both EtOH and atRA, as the amplitude of the potentiated EPSP remained significantly greater than the amplitude of the control EPSP in both conditions (EtOH: $p < 0.05$; atRA: $p < 0.001$; data not shown). Having shown that post-tetanic potentiation was maintained in both EtOH and atRA, I next examined whether the potentiated response changed over time. A mixed factor ANOVA was used to assess a change in post-tetanic potentiation over time in the presence of EtOH or atRA and showed no significant effect of either time or condition ($p > 0.05$) (Figure 24B). Thus, application of atRA did not significantly affect post-tetanic potentiation of this synapse.

Finally, I examined whether the maximum postsynaptic depolarization in RPeD1 produced by the tetanic stimulation of VD4 (Figure 22) changed significantly following application of atRA or EtOH (Figure 25). A mixed factor ANOVA was used to compare the maximum postsynaptic depolarization of RPeD1 in response to tetanic stimulation of VD4 in both EtOH and atRA. The results of the ANOVA revealed a significant effect of time ($F_{(3,27)} = 4.053$, $p = 0.017$), but again no significant interaction between time and condition. There were no significant differences in the response of RPeD1 in cell pairs exposed to EtOH compared to those exposed to atRA over 60 minutes. Interestingly, however, the total depolarization recorded in RPeD1 was significantly reduced in amplitude after 60 minutes of exposure to EtOH ($p < 0.05$; Figure 25), but did not significantly change following application of atRA. These findings suggest that there may be an effect of atRA on the transmitter release from the presynaptic cell (VD4) or on the summation of the EPSPs during the burst of action potentials, which prevented the significant decline in postsynaptic response over time. However, individual EPSPs during the compound depolarizing response were not clearly distinguishable in all cell pairs, which prevented me from examining this possibility in detail. I was thus unable to examine whether either synaptic facilitation or depression may have occurred during the depolarizing response in

RPeD1 in response to presynaptic tetanic stimulation, and future experiments will need to be designed with this in mind, in order to study this in more depth.

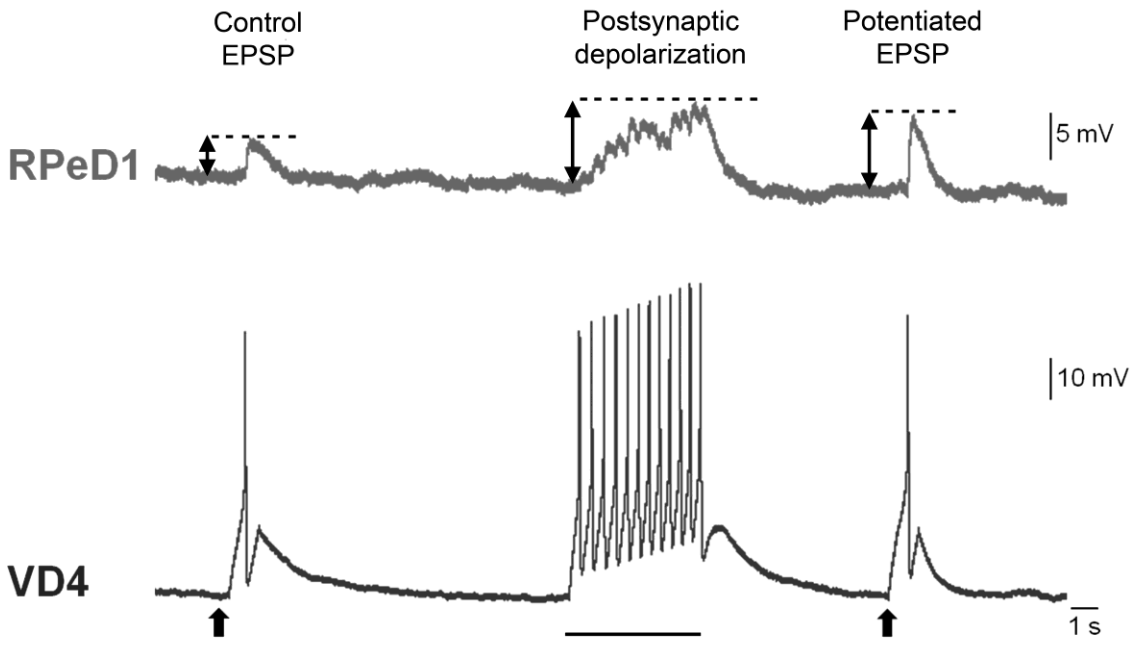

Figure 22. Synaptic potentiation between VD4 and RPeD1. Representative example of post-tetanic potentiation recorded from VD4 and RPeD1 after at least 18 hours in culture. The control EPSP is recorded prior to the burst of presynaptic activity in VD4. The postsynaptic depolarization refers to the compound EPSP response observed in RPeD1 as a direct result of the tetanic presynaptic activity. The potentiated EPSP is the first EPSP recorded shortly after the increase in presynaptic activity. The amount and duration of depolarizing current injection into VD4 was constant for each trial. Arrows indicate depolarizing current injection into VD4 to produce one action potential and the bar indicates the duration of depolarization to induce a train of action potentials.

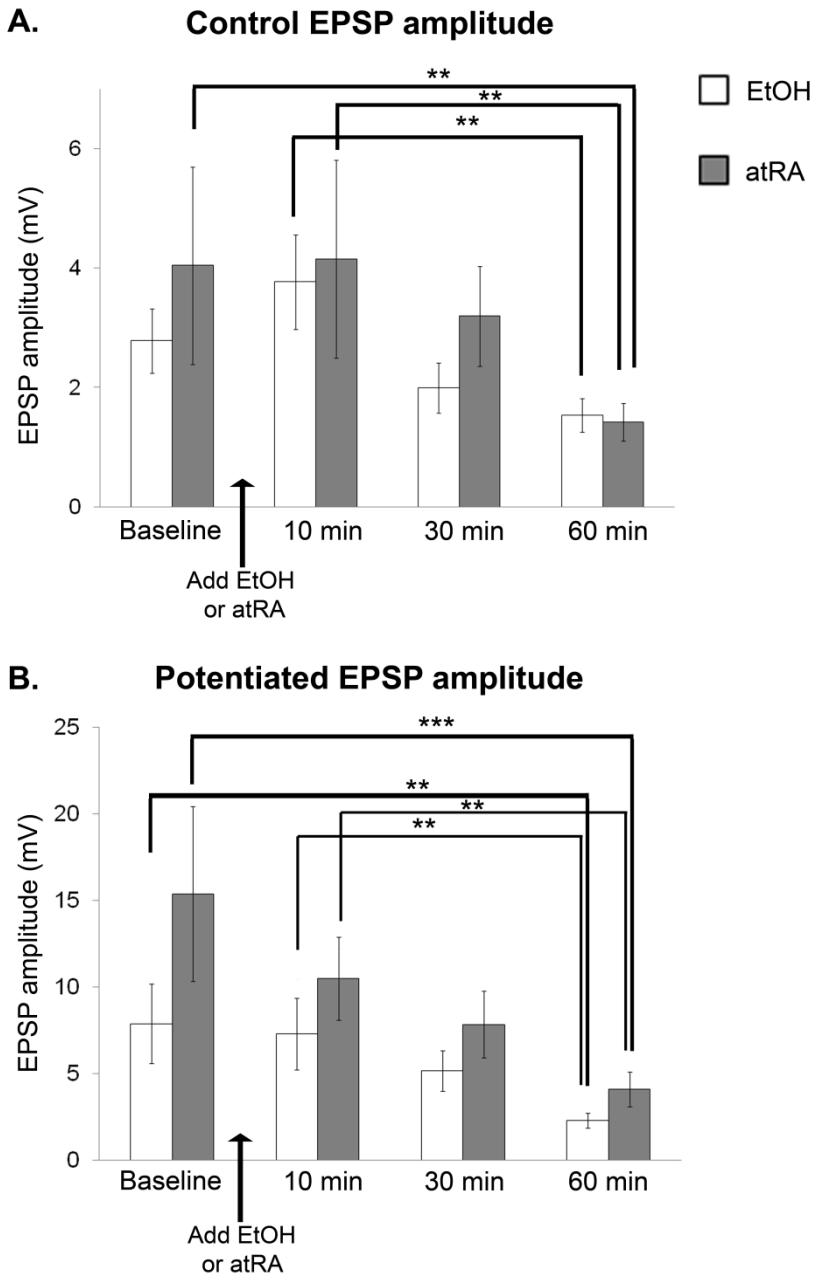

Figure 23. The amplitude of the EPSPs, both before and after tetanic stimulation of VD4, significantly decreased over time. (A) Following exposure to either EtOH (n = 5) or atRA (n = 6), the average control EPSP amplitude significantly decreased over 60 minutes. **(B)** The average potentiated EPSP amplitude significantly decreased over 60 minutes following exposure to EtOH (n = 5) or atRA (n = 6). The potentiated amplitude observed at 60 minutes was also significantly reduced relative to the EPSP observed after 10 minutes in EtOH or atRA. ** $p < 0.01$, *** $p < 0.001$.

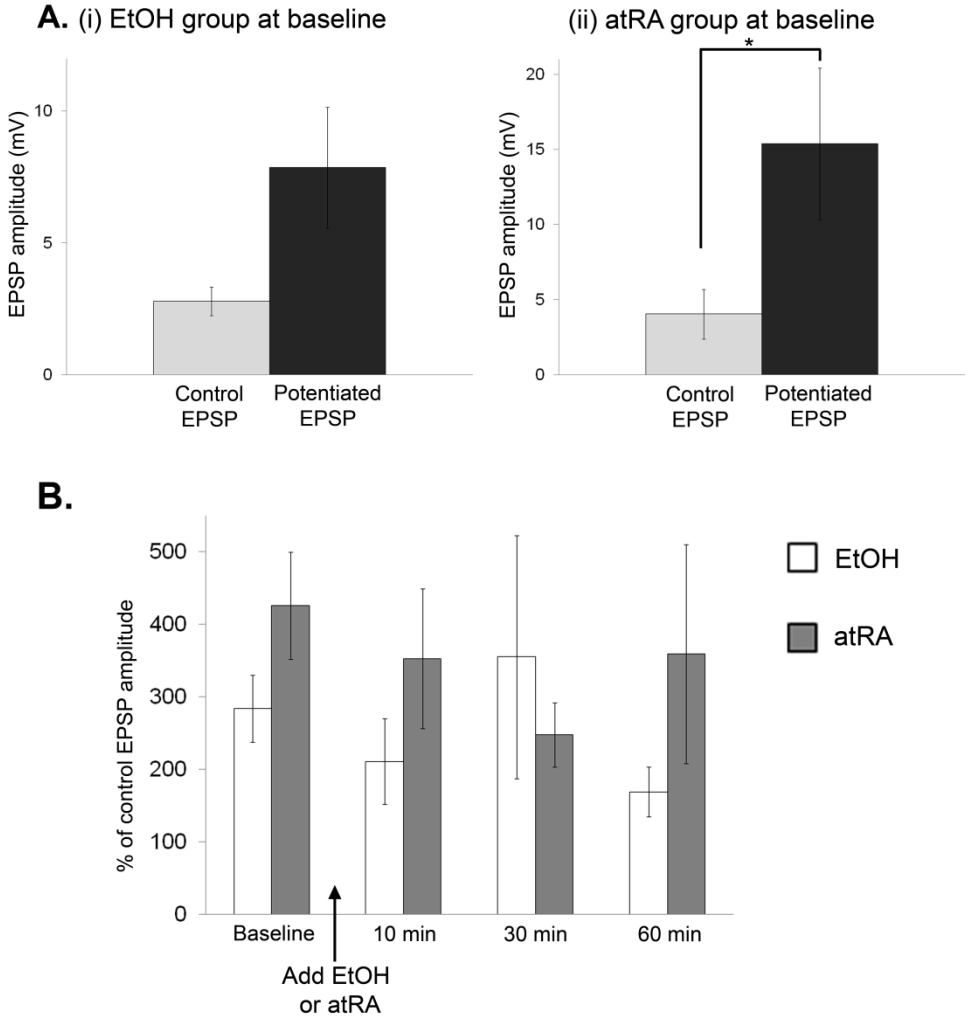

Figure 24. Post-tetanic potentiation does not significantly change over time. (A) Post-tetanic potentiation was verified at baseline prior to the application of **i)** EtOH (n = 5; p = 0.054) or **ii)** atRA (n = 6; p = 0.025). * $p < 0.05$ **(B)** Post-tetanic potentiation was determined by expressing the amplitude of the potentiated EPSP as a percentage of the amplitude of the control EPSP. Post-tetanic potentiation did not change significantly over time following application of EtOH or atRA.

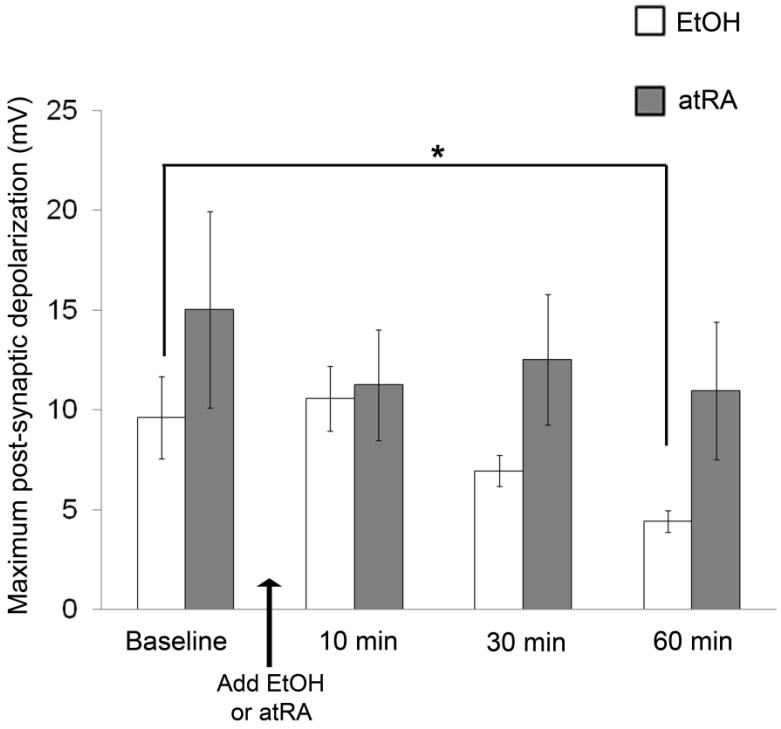

Figure 25. Maximum postsynaptic depolarization of RPeD1 is significantly reduced over time following exposure to EtOH, but not to atRA. The maximum depolarization observed in RPeD1 is significantly reduced following 60 minutes of exposure to EtOH (n = 5), but not following exposure to atRA (n = 6). However, no significant differences are reported between cell pairs exposed to EtOH and atRA over time. * $p < 0.05$

All-trans RA influences electrical synapses in a cell-specific manner

I showed above that acute application of atRA had no significant effect on post-tetanic potentiation of a chemical synapse in culture. However, since electrical synapses are also common in invertebrate CNS and RA is known to affect coupling of vertebrate gap junctions (Weiler et al., 1999; Zhang and McMahon, 2000), my next aim was to examine the effect of atRA on electrical synapses formed between molluscan neurons. I examined electrical synapses present between two different cell pairs: i) VD4 and RPeD2 and ii) VD4 and LPeD1. Though VD4 and RPeD2 (Syed and Winlow, 1989; Nesic et al., 1996) and VD4 and LPeD1 (Hamakawa et al., 1999) normally form chemical synapses, I observed strong electrical synapses between a number of these cell pairs in cell culture. Electrical synapses have been shown to precede chemical synapse formation (Todd et al., 2010) and, thus, the electrical synapses observed between these pairs may have been coupling as a result of the process of chemical synapse formation. I used these electrically coupled cell pairs to assess the effects of atRA on electrical coupling between molluscan neurons.

Electrical coupling between VD4 and RPeD2 is significantly reduced following application of retinoic acid

Intracellular recordings were made from electrically coupled VD4/RPeD2 cell pairs. Either atRA or EtOH was added to the bath solution to give a final bath concentration of 10^{-5} M atRA (Zhang and McMahon, 2000) or 0.1 % EtOH, and recordings were continued for another 60 minutes. A mixed factor ANOVA indicated a significant interaction between time and condition ($F_{(3, 39)} = 14.049$, $p < 0.001$) for electrical transmission from RPeD2 to VD4. Following application of atRA, electrical coupling from RPeD2 to VD4 (n = 8) was significantly reduced

by 30 minutes ($p < 0.01$), and coupling continued to decrease over the entire 60 minute period ($p < 0.001$). However, no change in electrical coupling was observed following application of EtOH ($p > 0.05$; $n = 7$; Figure 26Ci). At 60 minutes, coupling at synapses exposed to atRA was significantly reduced relative to synapses exposed to EtOH ($p < 0.05$). Bidirectional electrical coupling was observed in most pairs, and thus I also analyzed coupling from VD4 to RPeD2. A mixed factor ANOVA examining electrical coupling from VD4 to RPeD2 also revealed a significant interaction between time and condition ($F_{(3, 33)} = 8.053$, $p < 0.001$). Electrical coupling was significantly reduced 30 minutes following application of atRA ($p < 0.001$; $n = 6$) and continued to decrease over 60 minutes ($p < 0.001$), while no significant change was observed following the application of EtOH ($p > 0.05$; Figure 26Cii). At 60 minutes, electrical coupling at synapses exposed to atRA was significantly reduced compared to synapses exposed to EtOH ($p < 0.05$). These results are unlikely due to any changes in the input resistance of either VD4 or RPeD2. Raw values were used to ensure that input resistance did not change over time (at 60 minutes compared to baseline) for either cell in either EtOH or atRA (data not shown). The input resistance of VD4 measured at baseline was not significantly different from that measured after 60 minutes in EtOH (paired t-test; $p = 0.192$) or atRA (paired t-test; $p = 0.522$). Likewise, the input resistance of RPeD2 measured at baseline was not significantly different from that measured after 60 minutes in EtOH (Wilcoxon Signed Rank test; $p = 0.383$) or atRA (paired t-test; $p = 0.336$). The input resistance of VD4 at 60 minutes expressed as a percentage of baseline was 144.98 ± 21.52 % in EtOH and 112.68 ± 18.35 % in atRA, and these values did not differ significantly across conditions (t-test, $p = 0.275$). Likewise, the input resistance of RPeD2 at 60 minutes expressed as a percentage of baseline was 133.74 ± 32.25 % in EtOH and 87.46 ± 13.97 % in atRA, and these values were not significantly different across conditions (t-test; $p = 0.233$). Thus, a change in input resistance is unlikely to account for the results obtained in this study.

All-trans RA did not reduce electrical coupling between LPeD1 and VD4

Having observed that atRA significantly reduced coupling between RPeD2 and VD4, I next examined whether RA had the same effect on electrical synapses formed between LPeD1 and VD4. Once bidirectional electrical coupling between LPeD1 and VD4 was determined in a cell pair (Figure 27 A and B), atRA or EtOH were added to the bath solution to give a final bath concentration of 10^{-5} M atRA or 0.1 % EtOH, and synapses were monitored for another 60 minutes following drug application. A one-way repeated measures ANOVA showed no significant change in coupling from LPeD1 to VD4 ($F_{(3, 20)} = 0.761$, $p = 0.529$) or from VD4 to LPeD1 ($F_{(3, 20)} = 0.118$, $p = 0.949$) following application of atRA ($n = 8$ ($n = 7$ at 60 minutes)). As shown previously using RPeD2/VD4 synapses, application of EtOH ($n = 3$) did not significantly change electrical coupling at this synapse (LPeD1 to VD4: $F_{(3, 6)} = 0.621$, $p = 0.627$; VD4 to LPeD1: $F_{(3, 6)} = 1.146$, $p = 0.404$) (Figure 27C). Coupling observed 60 minutes after application of either EtOH or atRA did not significantly differ between groups ($p > 0.05$). As seen previously, the input resistance at 60 minutes (expressed as a percent of baseline values) did not differ between atRA and EtOH for either LPeD1 (atRA: 122.60 ± 11.02 %; EtOH: 122.82 ± 21.43 %) or for VD4 (atRA: 105.04 ± 13.45 %; EtOH: 100.64 ± 24.85 %; data not shown). Thus, atRA did not affect the input resistance of either cell differently than the vehicle control (EtOH). Taken together, these data suggest that atRA reduced transmission at electrical synapses formed between central neurons of *Lymnaea* in a cell-specific manner and this result is unlikely to be due to changes in the input resistance of the cells forming these synapses.

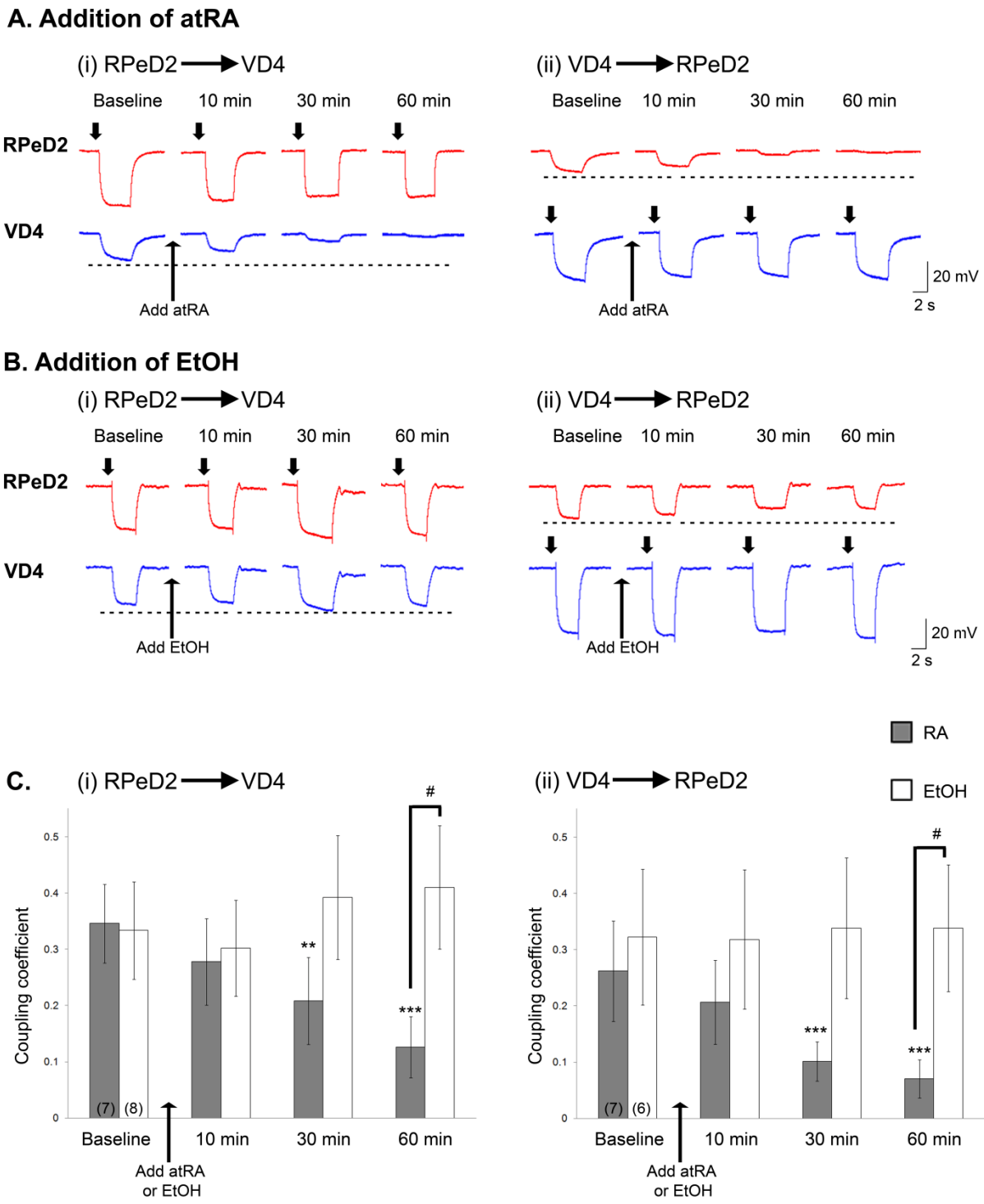

Figure 26. All-*trans* retinoic acid significantly reduced electrical coupling between RPeD2 and VD4. (A and **B)** Representative electrophysiological recordings from RPeD2 and VD4 before and following the application of atRA **(A)** and EtOH **(B)**. Arrows denote injection of hyperpolarizing current. **(C)** Application of atRA significantly reduced electrical coupling by 30 minutes, while EtOH had no significant effect on transmission at this electrical synapse. ** $p < 0.01$, *** $p < 0.001$ relative to baseline values; # $p < 0.05$ relative to EtOH control pairs. n values are noted on the graph.

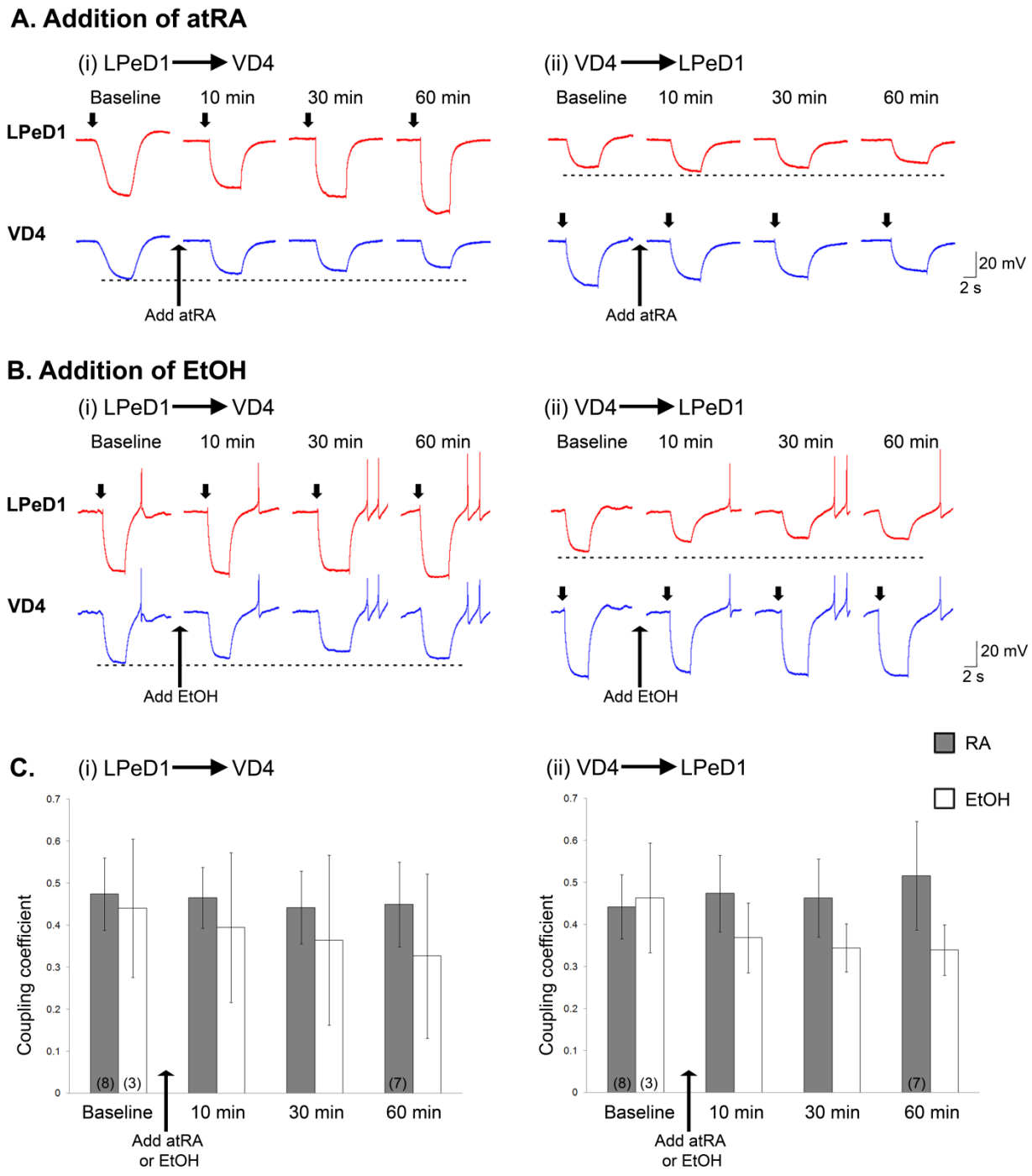

Figure 27. All-*trans* retinoic acid did not influence electrical coupling between LPeD1 and VD4. (A and **B)** Representative electrophysiological recordings from LPeD1 and VD4 before and following the application of atRA **(A)** or EtOH **(B)**. Arrows denote injection of hyperpolarizing current. **(C)** The bidirectional electrical coupling did not significantly change over time following the addition of either EtOH or atRA. n values are noted on the graphs.

4.05 Discussion

Behavioural changes as a result of learning and memory depend on synaptic plasticity (e.g. new synapse formation and changes in synaptic strength) within the CNS. Synaptic plasticity (e.g. LTP) is influenced by various neurotrophic factors, such as BDNF and NT-3, (Yamada et al., 2002; Shimazu et al., 2006). The role of RA as a trophic factor during synapse formation and/or modulation is not well known. However, its trophic role in neurite outgrowth has been demonstrated in both vertebrates (e.g. rat embryonic spinal cord (Wuarin et al., 1990); newt spinal cord (Dmetrichuk et al., 2005)) and invertebrates (e.g. cultured *Lymnaea* neurons (Dmetrichuk et al., 2006, 2008)). There is evidence to suggest that RA affects hippocampal plasticity (LTP and LTD) in rodents (Chiang et al., 1998; Misner et al., 2001; Nomoto et al., 2012) and can modulate synaptic transmission at vertebrate chemical (Aoto et al., 2008; Sarti et al., 2012) and electrical (Weiler et al., 1999; Zhang and McMahon, 2000) synapses. Together, these previous findings suggest that RA may influence learning and memory by affecting synaptic strength within the CNS.

I have previously demonstrated that retinoid signaling is important for LTM formation in *Lymnaea*. However, there is no previous evidence to indicate that RA affects synaptic formation and/or modulation in an invertebrate species. Therefore, in this study I determined whether RA might act as a trophic factor to influence synapse formation and/or synaptic transmission at chemical and electrical synapses formed between cultured, adult molluscan neurons. While the respiratory CPG consists of numerous chemical synapses (Syed et al., 1990), electrical synapses are not known to be directly involved in this network. However, electrical synapses are predominant within the *Lymnaea* CNS (e.g. electrically coupled neurons control the whole body withdrawal response; Syed and Winlow, 1991b) and may indirectly influence neuronal circuits

(Inoue et al., 1996). Thus, any influence of RA on transmission at electrical synapses may, in turn, influence other neural networks within the CNS.

In this study I was unable to determine whether RA exerted a trophic influence to induce excitatory synaptogenesis, as contrary to previously published results, excitatory synapses formed in the absence of extrinsic trophic factors. However, the excitatory synapses formed in the presence of atRA appeared to be, on average, stronger than those formed in the vehicle (control), although this difference did not reach statistical significance. Additionally, I did not see any evidence that RA influenced potentiation of a chemical synapse in cell culture. However, RA did reduce electrical coupling at synapses in a cell-specific manner. This study provides the first evidence that RA may modulate the strength of electrical synapses within an invertebrate nervous system.

Excitatory synaptogenesis occurred in the absence of extrinsic trophic factors

Previously published work indicates that inhibitory synapses reform in a soma-soma configuration in the absence of trophic factors (Feng et al., 1997), while excitatory synapse formation requires the presence of extrinsic trophic factors (Hamakawa et al., 1999; Woodin et al., 1999). I thus hypothesized that RA would act as a trophic factor to support excitatory synaptogenesis between *Lymnaea* somata. However, contrary to these previously published findings, I found that excitatory synapses formed in the absence of extrinsic trophic factors. While Meems et al. (2003) did report the formation of an excitatory synapse between the axon of LPeD1 and the soma of VD4 in 12 % of pairings in DM only, in that study an excitatory synapse was always observed in the presence of trophic factors. In my study, more excitatory synapses formed in the presence of RA (8 of 9) than in either control condition (DM: 3 of 9; EtOH: 5 of

10) on the plastic substrate. However, the proportion of excitatory synapses formed did not significantly differ across culture conditions. Thus, the trophic influence of RA was not necessary for excitatory synaptogenesis.

It is currently unclear why excitatory synapses formed in this study in the absence of trophic factors. It is possible that excitatory synapse formation may have been influenced by the presence of glia attached to the soma of LPeD1, which were visible in some (but not all) cell pairs. Glial cells can promote synaptogenesis in vertebrates (reviewed by Pfrieger, 2009); however, in *Lymnaea* the presence of glia previously prevented excitatory transmission between VD4 and LPeD1 in culture (Smit et al., 2001). This was attributed to an acetylcholine binding protein preventing acetylcholine (released from VD4) reaching the postsynaptic cell (LPeD1). While this previous finding does not support my hypothesis that glia are promoting synapse formation in my study, perhaps the differences can be explained by the different experimental procedures used. Smit et al. (2001) isolated glia from the pedal ganglia and placed them beside soma-soma pairs that had already been cultured, but the glia present in my study were already attached to LPeD1 when it was removed from the ganglia.

The formation of excitatory synapses in the absence of trophic factors could also be attributed to differences in the environmental conditions in which *Lymnaea* were bred and raised. Various environmental factors including crowding (De Caigny and Lukowiak, 2008), low calcium concentrations (Dalesman et al., 2011b), and temperature (Teskey et al., 2012) have previously been shown to influence memory formation (and consequently plasticity within the CNS). Thus, differences in environmental conditions may also affect the ability of these neurons to reform synapses in culture, and may have influenced excitatory synapse formation in my study, compared to previous studies.

Retinoic acid may prevent inappropriate synapse formation

While I could not determine whether RA acted as a trophic factor to induce excitatory synaptogenesis (as synapses did form in its absence), my data suggest that RA may have prevented the formation of inappropriate synapses between VD4 and LPeD1. Inappropriate inhibitory synapses have previously been reported to form between VD4 and LPeD1 in the absence of trophic factors (Woodin et al., 1999). In my study, inappropriate inhibitory synapses between VD4 and LPeD1 formed in both DM and EtOH when cells were cultured on the glass substrate. However, inappropriate inhibitory synapses never formed in the presence of RA. The number of cell pairs examined in each culture condition was, however, low and, thus, further study of this phenomenon would be required to confirm this preliminary finding.

Retinoic acid does not influence post-tetanic potentiation

Retinoic acid acts rapidly and nongenomically to influence homeostatic plasticity by enhancing excitatory transmission (Aoto et al., 2008) but suppressing inhibitory transmission (Sarti et al., 2013) between hippocampal vertebrate neurons. I, thus, investigated whether RA may also act rapidly to influence post-tetanic potentiation between invertebrate neurons.

My data showed a significant reduction in the amplitude of both the control EPSP and potentiated EPSP recorded in RPeD1, regardless of exposure to RA or its vehicle control (EtOH). However, there was no significant difference when comparing EPSP amplitudes recorded in EtOH with those in atRA, and this was true at each time point. From this, I can first conclude that RA did not prevent the synapse from weakening over time. While my data suggest an overall reduction in synaptic efficacy, post-tetanic potentiation was not observed to change significantly over time. Additionally, there was no significant difference in post-tetanic

potentiation between cells exposed to RA versus EtOH. However, it is possible that RA may exert an effect on synaptic function during tetanic presynaptic stimulation, as the maximum postsynaptic depolarization response recorded in RPeD1 was significantly reduced in the presence of EtOH over time, but not in the presence of atRA. However, I could not reliably distinguish the individual EPSPs during this depolarizing response in most cell pairs so I could not determine the contribution of facilitation or depression versus the temporal summation of individual EPSPs within the depolarizing response. Future experiments should be designed to generate more controlled stimulation of VD4 so that postsynaptic depolarization can be analyzed in more depth to determine whether RA could indeed be affecting synaptic transmission during a burst of action potentials in the presynaptic cell.

My finding that RA did not significantly influence post-tetanic potentiation of a chemical synapse may not be surprising given the outcome of my previous data. I have shown that retinoid signaling is required for LTM formation in *Lymnaea*, but not for learning or ITM. As only LTM (not ITM or learning) requires gene transcription (Sangha et al., 2003a), this suggests that RA may be exerting genomic influences and affecting gene transcription (either directly or indirectly), as opposed to rapid, non-genomic effects at synapses. Thus, examining the influence of RA over a 60 minute period may not reflect its potential to exert effects on gene transcription during LTM formation. I have shown preliminary evidence that chronic exposure to RA (at least 18 hours) may influence synaptic communication, as the EPSP amplitudes recorded in the presence of RA were on average larger than in the vehicle (EtOH) (though this difference did not reach significance). However, further experimentation will be required to study this in more detail.

Retinoic acid reduces electrical coupling in a cell-specific manner

While RA did not significantly influence potentiation of a chemical synapse, I found that it did affect transmission at electrical synapses. In this study I demonstrated a cell-specific effect of RA on the modulation of transmission at electrical synapses between cultured *Lymnaea* neurons. Unlike the previously published studies which assess the effects of RA on retinal neurons (Weiler et al., 1999; Zhang and McMahon, 2000), I used CNS neurons. I found that the acute application of all-*trans* RA significantly reduced electrical coupling between RPeD2 and VD4, but not between LPeD1 and VD4.

A change in the input resistance of a cell could influence the response of that cell either to the injection of current or to movement of current between cells, and might therefore affect the analysis of electrical connections. However, the input resistance of the cells used in this study did not change over time following the application of either EtOH or atRA. Furthermore, it has previously been shown that 10 μM RA does not change the input resistance of cultured *Lymnaea* Visceral F cells over 60 minutes (Vesprini and Spencer, 2014). Thus, the cell-specific effect of atRA on electrical coupling observed in this study is most likely due to a change in conductance via the electrical connections.

Retinoic acid has been shown to rapidly (and reversibly) decrease coupling between retinal horizontal cells in vertebrates (Weiler et al., 1999; Zhang and McMahon, 2000). This reduced coupling was also observed between retinal amacrine cells (Weiler et al., 1999), suggesting that the influence of RA on electrical coupling in vertebrates is not cell-type specific (at least within the retina). Zhang and McMahon (2000) proposed that an external RARβ/γ gates the junctional channels and is responsible for the rapid abolishment of coupling between the horizontal cells. While Zhang and McMahon (2000) propose the involvement of an RAR in the rapid modulation of electrical synapses, it may not be likely that the cell-specific effect observed

in this study can be attributed to the different expression of retinoid receptors in RPeD2 and LPeD1. *Lymnaea* likely only possess one RAR and one RXR and the distribution of both receptors is fairly widespread in the CNS (Carter et al., 2010, 2015). Thus, it is likely that RPeD2 and LPeD1 both possess both retinoid receptors, however this has not yet been confirmed.

The cell-specific effect could instead be related to the presence of different innexin isoforms in RPeD2 and LPeD1 which in turn may have different susceptibility to RA. In invertebrates, innexins have been shown to form intercellular channels. Invertebrate genomes can contain many different innexin genes, meaning that invertebrates can express a number of different innexins at individual gap junctions (Phelan and Starich, 2001). It is possible that RPeD2 expresses innexin isoforms that are susceptible to RA, while LPeD1 does not. However, the specific innexins present in *Lymnaea* are yet to be determined, and it is not yet possible to determine whether different innexins are expressed at these electrical synapses. A custom made antibody against an *Aplysia* innexin was tested on the *Lymnaea* CNS but did not cross-react, indicating that they do not possess the same innexin (C. Carter, personal communication). Thus, additional work is required in order to better understand the innexin composition of electrical synapses in the *Lymnaea* CNS.

The cell pairs used in this study to examine electrical coupling normally form chemical synapses. However, electrical coupling can precede chemical synapse formation (Todd et al., 2010), and so the strong electrical coupling I observed may have been a precursor to a chemical synapse. It has not yet been determined whether RA also affects electrical synapses between cultured cells normally showing electrical coupling *in vivo*. However, Vesprini (2006) showed that coupling between a different cell pair, RPD2 and VD1, in the isolated CNS was not significantly different following application of EtOH or RA. Future studies should thus

determine the susceptibility of normally electrically coupled cells (such as the Pedal A neurons) to atRA in cell culture.

While the cell-specific effect of atRA on electrical coupling is an interesting finding, it does not likely account for the impaired LTM observed in my studies, as the electrical synapse that responded to RA does not normally occur in the CNS or within the respiratory network. Although activity of an electrically coupled neuron (RPeD11) controls the whole-body withdrawal response and inhibits aerial respiration in *Lymnaea* (Inoue et al., 1996), the pneumostome stimulation used in my studies was insufficient to elicit this withdrawal response. Thus, the training procedure I employed is not likely to activate RPeD11 and no other electrical synapses that directly influence the respiratory network have been identified, though I cannot rule out that they may exist.

In summary, this study was the first to directly test the potential influence of RA on transmission at chemical and electrical synapses in an invertebrate (protostome) species. While I found no evidence to indicate that RA influenced post-tetanic potentiation of a chemical synapse, the amplitude of EPSPs produced at the excitatory synapses formed in atRA were on average larger than those formed in the vehicle control (though this difference was not statistically significant). Additionally, I have shown that RA reduced transmission at electrical synapses in a cell-specific manner which suggests that RA may indeed influence synaptic plasticity in an invertebrate species.

Chapter 5:

Conclusions and Perspectives

The overall aim of this thesis was to investigate whether RA, the active metabolite of vitamin A, is required for learning and memory formation in an invertebrate species. While retinoid signaling is known to be important for hippocampal-dependent memory (Chiang et al., 1998; Cocco et al., 2002; Bonnet et al., 2008), no previous studies have shown a role for RA in implicit memory formation in an invertebrate species. In this thesis I demonstrated that when retinoid signaling was impaired in *Lymnaea*, learning and ITM formation occurred, but LTM formation did not. Interestingly, activation of the retinoid receptors using various agonists resulted in the promotion of LTM. I also aimed to examine whether RA promoted memory formation, but this proved difficult because the photosensitive nature of RA required incubations to be conducted in darkness. This led to the observation that darkness alone (in the absence of RA) promoted LTM formation, and the data suggested that this may have occurred as a result of interactions with the retinoid signaling pathway.

Synaptic plasticity is a neural mechanism underlying memory formation and is influenced by various trophic factors. I therefore hypothesized that RA may exert a trophic effect to influence synaptic plasticity within the *Lymnaea* CNS. I did not find any evidence to indicate that RA influences post-tetanic potentiation of a chemical synapse between *Lymnaea* neurons. However, RA did reduce transmission at electrical synapses, albeit in a cell-specific manner.

Retinoid signaling is conserved between vertebrates and invertebrates

Retinoid signaling is important in both the developing and adult vertebrate nervous system (Maden, 2007). However, RA has also emerged as an important molecule in various invertebrate species, as it influences embryonic development (Carter et al., 2010, 2015), limb regeneration (Hopkins and Durica, 1995; Hopkins, 2001), and neuronal outgrowth and survival

(Dmetrichuk et al., 2006, 2008). The genetic machinery required for retinoid signaling has been identified in both non-chordate deuterostome and protostome invertebrate species (Cañestro et al., 2006; Albalat and Cañestro, 2009). Endogenous retinoids have also been detected in invertebrates including the insect *Locusta migratoria* (9-*cis* and all-*trans* RA; Nowickj et al., 2008) and the gastropod molluscs *Osilinus lineatus* (retinyl esters; Gesto et al., 2012) and *Lymnaea stagnalis* (9-*cis* and all-*trans* RA; Dmetrichuk et al., 2008). Additionally, Gesto et al. (2012) have provided evidence for the conversion of retinaldehyde to RA in *O. lineatus* and RALDH activity has been demonstrated in *Lymnaea* CNS extracts (Dmetrichuk et al., 2008).

Invertebrate retinoid receptors have also been shown to bind and be activated by RA. The RXRs present in the jellyfish *Tripedalia cystophora* (Kostrouch et al., 1998), the fiddler crab *Uca pugilator* (Hopkins et al., 2008) and the locust *L. migratoria* (Nowickj et al., 2008) bind 9-*cis* RA. Additionally, an RXR from the mollusc *Biomphalaria glabrata* induces gene transcription following exposure to 9-*cis* RA (Bouton et al., 2005). However, the locust RXR also binds all-*trans* RA with an affinity similar to that of 9-*cis* RA (Nowickj et al., 2008), and exposure to all-*trans* RA upregulates the expression of RXR mRNA in the marine sponge *Suberites domuncula* (Wiens et al., 2003). Thus, invertebrate RXRs may be less selective for RA isomers than vertebrate RXRs (which demonstrate a preference for 9-*cis* over all-*trans* RA), and it is possible that both 9-*cis* and all-*trans* RA serve as natural ligands for the RXR in invertebrates.

However, not all invertebrate retinoid receptors bind with RA. In the rock shell *Thais clavigera*, the RXR isoforms are activated in the presence of 9-*cis* RA (Urushitani et al., 2011), but the RAR does not appear to be activated by all-*trans* RA (Urushitani et al., 2013). Thus, the presence of a retinoid receptor does not guarantee that it functions the same way in invertebrates as in vertebrates. While both an RAR and RXR have been identified in *Lymnaea*, and both 9-*cis*

and all-*trans* RA have been detected within the CNS and hemolymph (Dmetrichuk et al., 2008), it has not yet been determined whether these receptors bind either ligand or show a preference for one ligand over another. However, the *Lym*RXR shares approximately 97 % amino acid identity with the RXR of the mollusc *B. glabrata* which is known to bind 9-*cis* RA (Bouton et al., 2005; Carter et al., 2010). Thus, it seems likely that the *Lym*RXR will also bind RA.

The role of retinoid signaling in invertebrate memory formation

In these studies the retinoid signaling pathway was targeted using both retinoid receptor antagonists and agonists. I have demonstrated that targeting either the RAR or the RXR influenced memory formation in this mollusc, suggesting that both receptors may be involved in this process. This agrees with vertebrate studies which demonstrated that RARβ/RXRγ double mutants consistently showed impaired spatial memory (Chiang et al., 1998; Wietrzych et al., 2005). The agonists and antagonists used in this thesis are designed for vertebrate receptors, and their selectivity for the *Lym*RAR and *Lym*RXR has not yet been confirmed. However, they have previously been shown to be effective in this mollusc (Carter et al., 2010; Rand, 2012; Vesprini and Spencer, 2014).

Interestingly, data from our lab demonstrate that retinoid signaling is also important for the formation of LTM following classical conditioning of the feeding behaviour. In collaboration with D. Britt and N. Patel, we determined that impairing RALDH enzyme activity prevents *Lymnaea* from forming LTM following training with a classical conditioning procedure, further supporting the findings of this thesis. This suggests a general role of RA in memory formation, regardless of the nature of conditioning or the neural networks involved. However, both training paradigms used in these experiments were aversive in nature and, thus, it would be interesting to

examine whether retinoid signaling is also required for memory formation following a nonaversive training procedure.

One advantage to studying memory formation in *Lymnaea* is that the neural networks underlying both the aerial respiratory behaviour and the feeding behaviour have been mapped and studied (Benjamin et al., 2000). Thus, it is possible to directly examine neural correlates of memory formation in individually identified neurons, which is not feasible using most vertebrate preparations. While I have demonstrated that RA is necessary for LTM formation in *Lymnaea*, other model invertebrates (such as *Drosophila melanogaster* and *Caenorhabditis elegans*) used for studying memory formation do not appear to possess the full retinoid signaling machinery. Therefore, *Lymnaea* is an ideal candidate to examine specific RA-induced changes within underlying neural networks. My demonstration of a role for RA in memory formation in a protostome supports an ancient role for this molecule in memory. However, the apparent lack of a fully functioning retinoid pathway in other protostomes indicates that retinoid signaling is unlikely to be necessary for memory formation in all protostome species. It is likely that other trophic molecules exist that replace the function of RA and produce the same end result in these species lacking retinoid signaling machinery.

Studies in vertebrates have focused on the role of retinoid signaling in the formation of hippocampal-dependent memories, such as spatial memory (Chiang et al., 1998; Wietrzych et al., 2005). With these studies, I have provided evidence that retinoid signaling is also involved in the formation of implicit memory following operant conditioning in an invertebrate species. Thus, the role of RA in memory formation appears to be conserved between vertebrates and invertebrates as well as across different memory systems (i.e. explicit and implicit memory). The involvement of RA in different forms of memory may be the result of its classical role in regulating gene transcription; RA interacts with retinoid receptors which bind to RAREs on

target genes and induce transcription. It is possible that RA targets the same genes during the formation of different types of memory; for instance, RA may target specific genes important for synaptic plasticity and/or memory formation regardless of the memory system invoked.

Where is retinoic acid acting during long-term memory formation?

Following operant conditioning, *Lymnaea* form implicit memory which is believed to be stored within the neuronal network controlling the conditioned behaviour (Kandel et al., 2014). Thus, the memory of the conditioned response in these studies is likely stored within the CPG underlying aerial respiration. I have shown that impairing RALDH activity prior to, but not following, operant conditioning impairs LTM formation, but does not affect learning or ITM formation. This correlates with the finding of Scheibenstock et al. (2002), that soma ablation of RPeD1 prior to training does not impair learning or ITM formation, but prevents LTM, while ablation following training had no effect. Scheibenstock et al. (2002) hypothesized that gene transcription within the soma of RPeD1 is responsible for LTM formation. Thus, perhaps RA is acting via its classical role to regulate gene transcription within RPeD1 to influence LTM formation. However, network changes following conditioning occur throughout the respiratory CPG (Spencer et al., 1999) and, thus, memory impairment resulting from impaired retinoid signaling may reflect differences in activity within any of the CPG neurons.

In this study, I examined whether RA influences synapse formation and/or synaptic plasticity, in order to specifically determine the trophic capabilities of RA at *Lymnaea* synapses (which have not previously been examined). Not all synapses studied are components of the respiratory CPG and thus may not contribute directly to network changes occurring during or following operant conditioning of respiration. The connection from VD4 to RPeD1 is, however,

a component of the respiratory CPG and I studied the effects of RA on potentiation at this synapse. However, this connection is biphasic (though predominantly inhibitory) in the intact CNS, whereas I studied the excitatory component of this synapse. As the role of the excitatory component is not well defined, I am unable to determine its influence on network changes following conditioning. Even though the other synapses studied are not components of the respiratory CPG, the presynaptic cell in all pairs was VD4, which controls pneumostome closing. Thus, this study may provide insight into how RA may affect the efficacy of connections made by this important CPG neuron (both chemical and electrical), which may ultimately affect network properties dependent on the activity and/or connections of this neuron.

Future studies should examine the site of action of RA using electrophysiological recordings made from the CNS of *Lymnaea* treated with RALDH inhibitors or retinoid receptor antagonists. The electrophysiological activity in the CNS of experimental animals can then be compared to the activity observed in animals treated with the vehicle and pond water control animals. Since certain neural correlates of LTM formation have already been identified (Spencer et al., 1999; Khan and Spencer, 2009), any differences observed between control and experimental animals immediately following the memory test may provide insight into which cells and network properties are affected by RA.

Is retinoic acid acting via its classical genomic role in LTM formation?

Retinoic acid classically acts to regulate gene transcription by binding to nuclear RXRs and RARs (Maden, 2007). In this thesis, I demonstrated that retinoid signaling is important for LTM, but not learning or ITM formation. Since LTM requires gene transcription while learning and ITM do not (Sangha et al., 2003a), this suggests that RA may be acting via its classical role

to influence gene transcription during LTM formation. However, both the *Lym*RXR and *Lym*RAR have a non-nuclear distribution in the *Lymnaea* CNS and have been localized to neurites of regenerating adult *Lymnaea* neurons in culture (Carter et al., 2010, 2015). Thus, these receptors may not be acting directly as transcription factors during LTM formation in *Lymnaea* and may, instead, exert nongenomic effects during memory formation. Retinoic acid has been shown to influence presynaptic neurotransmitter release (Liao et al., 2004) as well as dendritic spine formation (Chen and Napoli, 2008) in a non-traditional, nongenomic manner. Additionally, RA nongenomically modulates synaptic transmission at both excitatory and inhibitory chemical synapses between vertebrate hippocampal neurons (Aoto et al., 2008; Sarti et al., 2012). Vesprini and Spencer (2014) have shown that acute application of atRA nongenomically influences the firing properties of *Lymnaea* neurons (and isolated neurites) in cell culture. Of interest to this thesis is the observation that acute application of atRA can silence RPeD1 (the neuron that initiates aerial respiration), as well as isolated RPeD1 neurites, suggesting that RA may reduce the firing activity of this important CPG neuron (Vesprini and Spencer, 2014). Interestingly, RPeD1 becomes more silent following the operant conditioning of respiration, compared to yoked controls (Spencer et al., 1999). It is therefore possible that RA nongenomically reduces the activity of RPeD1 during its role in LTM formation. Indeed, artificially silencing RPeD1 for only 20 minutes has been shown to induce LTM formation using only an ITM conditioning procedure (Lowe and Spencer, 2006); notably this result is very similar to that achieved with the retinoid receptor agonists used in my study. Alternatively, RA may nongenomically activate other signaling pathways such as CREB (cAMP response element-binding protein; Cañón et al., 2004), which may lead downstream to the transcription of target genes (as shown during LTP). Thus, in summary, it is quite possible that RA might act via a combination of genomic and nongenomic mechanisms during the formation of LTM in *Lymnaea*.

It is also possible that the operant conditioning procedure itself may induce changes in retinoid signaling. If RA is acting genomically to influence gene transcription underlying LTM formation, perhaps the retinoid receptors translocate from the cytoplasm to the nucleus in response to the aversive operant conditioning procedure (as both receptors have a non-nuclear expression within the CNS; Carter et al., 2010, 2015). Schrage et al. (2006) have shown the translocation of RARα, RXRα, RXRβ, and RXRγ from the cytoplasm to the nucleus following spinal cord injury in rats (as unlesioned samples show a mostly cytoplasmic distribution). A future goal should be to determine if aversive conditioning paradigms cause retinoid receptor translocation to the nucleus in *Lymnaea*. Additionally, the training procedure itself may change the mRNA expression of retinoid signaling machinery which in turn may reflect an increase in protein expression. J. Simmons has shown with qPCR that following operant conditioning, the relative expression of Cyp26 mRNA is decreased compared to naive, untrained animals (personal communication). If this is indeed the case, a reduction in Cyp26 expression would likely lead to increased RA levels. However, no change was seen with RALDH expression (neither RAR nor RXR expression was examined). This indicates that the gene expression within the *Lymnaea* CNS changes following operant conditioning. Future work examining RAR and RXR expression in naive and operantly conditioned snails will provide insight into how the retinoid signaling pathway may be modulated by the training procedure itself.

Taken together, the data presented in this thesis support an ancient role for retinoid signaling in memory formation and provide a basis for future studies into how RA may be involved in the neural correlates of learning and memory in an invertebrate species.

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